Development of Transgenic *Ambystoma mexicanum* (axolotl) to Study Cell Fate during Development and Regeneration

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SUMMARY

The establishment of transgenesis in axolotls is crucial for studying development and regeneration, as it would allow for long-term cell fate tracing as well as gene expression analysis, therefore we were interested in both obtaining animals expressing the transgene with little mosaicism in F0 generation and transgenesis. We demonstrate here that plasmid injection into the one cell stage axolotl embryo generates transgenic animals that display germline transmission of the transgene. However, the efficiency of simple plasmid injection is very low, expression of the transgene is mosaic and seems to be promoter dependant.

We have tested several methods of transgenesis developed in other systems. First we used Adeno-associated Viral Inverted Terminal Repeats inserted into the injected construct to enhance the expression level of the transgene and reduce mosaicism (Fu et al., 1998). However, in the axolotl system we do not observe the enhancement of expression. Moreover, the expression appeared to be transient and disappeared after two months.

Further, we tested the effect of inclusion of *SceI* meganuclease in the injections, successful transgenesis method in the medaka system (Thermes et al., 2002). It resulted in a higher percentage of F0 animals displaying strong, stable expression throughout the body. This represents the first demonstration in the axolotl of germline transmission of a transgene. Using this technique we have generated a germline transgenic animal expressing GFP ubiquitously in all tissues examined.

We have used this animal to study cell fate in the dorsal fin during development. We have discovered a contribution of somite cells to dorsal fin mesenchyme in the axolotl, which was previously assumed to derive solely from neural crest.

We have also studied the role of blood during tail regeneration by transplanting the ventral blood-forming region from GFP+ embryos into unlabeled hosts. During tail regeneration, we do not observe GFP+ cells contributing to muscle or nerve, suggesting that during tail regeneration blood stem cells do not undergo significant plasticity.

We are interested in characterization of a pluripotency of blastema cells. Previously, it has been shown that neural progenitor cells from the spinal cord can transdifferentiate to a muscle and other tissue types in the regenerating tail (Echeverri and Tanaka,

2002a). To test if blastema cells have the potency of differentiating into a neural tissue, we transplanted GFP+ 4 day blastema into an injured spinal cord. Our result shows that blastema cells don't seem to contribute to the regenerating spinal cord.

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1 GENERAL INTRODUCTION

1.1 Introduction – on the importance of transgenesis

The production of transgenic animals is a powerful tool used successfully in many animal systems to study early embryogenesis, organogenesis and adult tissues. It would be a particularly important technique for studying regeneration, as it would allow for long-term cell fate tracing, as well as gene expression analysis, two important aspects of understanding regeneration on a mechanistic level. Vertebrate regeneration is studied in several different model organisms, including caudata (salamanders) such as *Ambystoma mexicanum* (axolotl), anurans such as *Xenopus laevis*, and teleosts such as *Danio rerio* (zebrafish) and *Oryzias latipes* (medaka). Each of these systems has distinct features for studying regeneration and these distinctions make it crucial to have transgenic and genomic resources available in each of the organisms. For example, *Xenopus* is able to regenerate its limbs and tails as a tadpole but then loses this capacity upon metamorphosis while salamanders retain the ability to regenerate a wide variety of tissues and structures including muscle, cartilage, skin, spinal cord, lens and jaw throughout their lifespan (Goss, 1969).

A number of molecular and cellular aspects of regeneration are also distinct. In particular, wider plasticity of cell differentiation is observed during regeneration in caudata compared to *Xenopus*. Implantation and lineage tracing experiments have shown that in caudates, dedifferentiation of multinucleated muscle fibers into proliferative mononucleate cells contributes a significant number of cells to the blastema (Echeverri et al., 2001; Kumar et al., 2000; Lo et al., 1993). Furthermore *in vivo* tracing of spinal cord cells revealed that neural progenitors have the potential to switch fate and form muscle and cartilage during tail regeneration.(Echeverri and Tanaka, 2002a). So far, it has been accepted that differentiated state of the cells is rather fixed and an unchanging attribute. In this work spontaneous metaplasia *in vivo* was observed, where one type of a differentiated cell undergoes changes to form another differentiated cell. Such metaplasia was not observed in *Xenopus* cell tracking experiments where each tissue layer exclusively regenerated itself (Gargioli and Slack, 2004).

While teleosts are able to regenerate their fins and portions of the heart, complete regeneration of the spinal cord, as observed in axolotls, is not possible in most teleost species (Kawakami et al., 2004). On a cellular level the plasticity of cell differentiation during teleost regeneration has not yet been widely established.

Work in *Xenopus laevis* provides an excellent example of how transgenesis can be used for studying regeneration. The Cre-loxP system was used to trace muscle cell fate during tail regeneration where it was shown that muscle fibers do not dedifferentiate (Gargioli and Slack, 2004; Ryffel et al., 2003). Gargioli and Slack also grafted embryonic tissue from GFP+ *Xenopus* embryos into unlabelled hosts to follow the fate of other tissue types and found no transdifferentiation of spinal cord and notochord cells during tail regeneration. To address the molecular pathways involved in initiating regeneration Beck and colleagues (Beck et al., 2003; Beck et al., 2001) created transgenic animals where key extracellular and intracellular signaling molecules were under the control of the *Xenopus* HSP70 promoter, allowing induction of gene expression at the time of tail amputation. This work implicated a role for the Notch and BMP pathways in induction of tail regeneration. These *Xenopus* studies illustrate the value of transgenic approaches for regeneration research. As the cellular and molecular differences between tail regeneration in anurans and caudata becomes more evident, the significance of studying regeneration in both taxa is becoming increasingly clear.

1.2 Overview of selected methods of gene delivery.

The ability to selectively alter gene expression creates enormous possibilities in regeneration studies. Finding what pathway is a key player in inducing and guiding regeneration is an ultimate goal in regeneration research. It is, therefore, of particular importance to develop transgenic approaches in caudata.

For this purpose it is necessary to establish a nomenclature of transgenesis. Germline integration is an introduction of exogenous DNA into the host genome and its stable integration the host genome, transmitted to the second generation. Transgenesis is an introduction of exogenous genes into the host organism, its stable integration in the genome and transmission to the next generation (germline integration) and proper expression of this transgene in the host organism. (Grabher et al., 2004). For our studies of tracking cell fate in the tail regeneration we are interested in obtaining the level of transgenesis with properly expressed transgene in the second generation.

The axolotl is an animal easily bred under laboratory conditions with a generation time of approximately one year. An advantage of using axolotls is the availability of skin pigment mutants such as the *white* mutant where pigment cells are inhibited from migrating out laterally under the epidermis from their neural crest origin on top of the neural tube (Epperlein and Lofberg, 1990). These mutants facilitate visualization of lineage tracers such as GFP in regeneration studies (Echeverri and Tanaka, 2002b; Echeverri and Tanaka, 2003). Our goal was to establish an efficient transgenesis method for the axolotl that would allow first, production of a large number of transgenic animals in the F0 generation and second, germline transmission.

There are several methods of introducing the transgene into the genome available in various vertebrate systems. The most efficient so far was modified sperm nuclei transplantation (REMI) into the oocyte in the *Xenopus* system (Kroll and Amaya, 1996). This method of transgenesis results in stable, nonmosaic expression of cloned genes in the embryos. It is possible in *Xenopus* to produce a large number of transgenic, normally developing embryos that express transgenes at high frequencies – most importantly there is no need to wait for a germline transmission in F1 to obtain transgenics.

In systems like axolotl, where generation time is one year long possibility of applying this method and obtaining such a tool in F0 generation would be a mile stone. Since 1996, there were attempts to simplify this complicated and technically demanding method (Sparrow, 2000). Ueda and colleagues (Ueda et al., 2005) successfully applied a modification of the *Xenopus* transgenic technique involving *in vitro* fertilization coupled to sperm injection to produce transgenic newt, *Cynops pyrrogaster*. Transgenic animals were generated at a frequency of approximately 2% and displayed uniform transgene expression throughout the body. Unfortunately, a disadvantage of the *Cynops* system is the complex life cycle and long time to sexual maturity, making propagation of transgenic lines complicated. Furthermore, in the Ueda protocol a large number of females were required to produce enough unfertilized eggs for a single experiment, making the possibility of producing multiple transgenic lines difficult. Another transgenic approach that also has been described for the newt (Makita et al., 1995) found that injection of fertilized *Cynops* eggs with a linearized eukaryotic expression vector containing the lacZ gene resulted in mosaic reporter gene expression in tailbud stage embryos. The inclusion of newt satellite 2 sequences in the plasmid augmented the extent of expression 10-fold, with a widespread distribution of gene expression in hatching larvae. Germline transmission was not assessed in these experiments.

In contrast to previous methods, which are quite complex, there were several simpler protocols of producing transgenic animals, and three of them were considered for application in the axolotl system.

The plasmid injection into the one cell stage embryo is the simplest method used in zebrafish and *Xenopus* (Cleaver and Krieg, 1999; Stuart et al., 1988). It is easy and requires only cloning of the desired construct. However, a disadvantage of this method is a very low efficiency of transfection, germline transmission and transgenesis. In most cases the transgene remains episomal and its expression is mosaic, transient and weakens in time - probably due to inefficient segregation of the transgene during development. Moreover, it is very difficult to obtain tissue specificity of gene expression.

There are two other methods which use plasmid injection in combination with factors enhancing integration of the transgene into the host genome and increasing possibility of transgenesis. One is the application of the Adeno-associated Viral Inverted

Terminal Repeats (Fu et al., 1998). The Adeno- associated virus is a non-autonomous parvovirus requiring co-infection with another virus for productive infection and is completely nonpathogenic (Gaiano and Hopkins, 1996). It contains the terminal repeat sequences of 145 nucleotides, which form a palindromic hairpin structure that is required for DNA replication, integration and eventual excision of proviral sequences. To use the ability of integrating into the host genome of the adenovirus, it is necessary to build a vector where the desired expression cassette is flanked by ITR hairpins. As reported for *Xenopus*, inclusion of AAV-ITRs in DNA plasmids significantly increases transgene expression from a ubiquitous promoter, reduces ectopic expession and significantly enhances tissue specific expression (Fu et al., 1998). This method gives results resembling the efficiency and fidelity of the sperm nuclear transplantation technique (Kroll and Amaya, 1996) and is simpler, however the transgenesis could be obtained only in F1 generation.

A second method very successfully tested in the zebrafish system is the use of *ISceI* meganuclease enzyme (Thermes et al., 2002). *ISceI* meganuclease is a site specific endonuclease, encoded in the intronic sequence, originally discovered in *S. cerevisiae* (Belfort and Roberts, 1997). The enzyme is able to recognize and cleave an intronless allele and introduce the DNA fragment into the cleavage site (Colleaux et al., 1988; Jacquier and Dujon, 1985). The recognition sequence of the *ISceI* enzyme is 18 base pairs long with a very high specificity of recognition. The site is expected to be found only once in $7x10^{10}$ bp of random sequence and until now it hasn't been found in any vertebrate genome. Therefore it is very unlikely that fractionation of the host genome will occur as it happens with the REMI technique (Kroll and Amaya, 1996). Co-injection of the ISceI enzyme together with the plasmid containing its recognition sites resulted in enhanced promoter dependant expression of the transgene in F0 generation and a significant increase in the number of expressing embryos and also in the number of cells expressing the transgene per embryo. Moreover, animals showed reduction of mosaicism and the frequency of transgenesis increased at least two fold compared to control injections (Thermes et al., 2002).

After analyzing the available methods, we decided to establish a microinjection protocol using a plasmid with an universal promoter driving GFP, and further test both methods transgenesis enhancement – AAVITRs and *ISceI* meganuclease enzyme.

1.3 Transgenic GFP axolotl in development and regeneration studies

The availability of a GFP transgenic animal is a valuable tool for cell tracking experiments, since it provides an indelible marking system with high spatial resolution that is visible in live animals. These animals provide a transplantation system comparable to the valuable quail-chick chimeras, which have been crucial for studying cell fate during avian embryogenesis and organogenesis (N.M. LeDouarin, 1999). The axolotl is a particularly good system for tracking cell fate by transplanting tissues from a GFP+ animal into unlabeled hosts due to its unrivaled accessibility for tissue engraftment at any stage of its lifecycle. The transplantation of GFP+ cells circumvents the problems of other labeling techniques such as DiI, where the possibility of the dye spreading to unwanted cells always exists, or mRNA/dextran injection and electroporation which are relatively transient markers that are diluted out upon cell division.

Here we use a GFP transgenic axolotl in embryonic tissue grafting to study three essential problems in development and regeneration. By transplantation of neural folds and somites, we demonstrate that the dorsal fin mesenchyme, which was originally assumed to derive entirely from neural crest (see (Tucker and Slack, 2004), has a dual origin, deriving from both from neural crest and somites.

Second, we examine the issue of hematopoietic cell plasticity and its contribution to regeneration. Recent reports of hematopoietic cells being able to contribute to other tissue lineages such as muscle or brain in the mouse have raised controversy (Brazelton et al., 2000; Ferrari et al., 1998; Ferrari and Mavilio, 2002; Mezey et al., 2000). The frequency of such events, as well as their potential relevance to tissue regeneration are still open to question. We wanted to test whether blood cells display widespread plasticity during axolotl tail regeneration—namely whether blood cells contribute significantly to muscle and the central nervous system. Here we do not observe blood cells contributing to muscle or the central nervous system during tail regeneration, indicating that hematopoetic cell plasticity is not an ubiquitous mechanism used during regeneration.

A spinal cord during axolotl tail regeneration grows as a separate entity, however it has been shown that its cells can form other types of cells in the regenerating tail

(Echeverri and Tanaka, 2002a). We attempt to reveal whether blastema cells have the potential to contribute to the regenerating spinal cord. We transplant GFP+ blastema into the injured spinal cord. We observe the contribution of blastema cells to a cartilage and muscle, however in each case blastema cells were pushed away from the spinal cord region and did not contribute to this structure.

2 RESULTS

2.1 Introduction

The axolotl system is a great model for studying regeneration. The ability of the animal to regenerate its lost body structures is maintained throughout the life. The regeneration of the tail raises numerous questions about how it is possible to reconstitute such a complicated structure, where not only the morphology but also its full function is recovered. For example, does the process of regeneration use the same pathways as development? What is the origin of the blastema? Is the blastema a pool of stem cells normally residing in the tissue that are activated upon injury or do these stem cells come form dedifferentaiation? Are blastema cells pluripotent or do they have a restricted fate?

There have been several reports on the origin of blastema cells (Echeverri et al., 2001; Echeverri and Tanaka, 2002b; Muneoka et al., 1986). However, in all of them authors used dyes, transient expression of markers or a triploid/diploid cell marker. The use of dye (e.g. rhodamin dextran) to label cells allows only short-term fate tracking because cells lose the marker upon proliferation due to dilution of the dye. It is also not possible to exclude that dye can pass to another cell – a problem difficult to overcome while tracking the fate of a single cell.

The transient expression of markers in the axolotl is very ineffective in long-term cell tracking because the expression is lost upon dedifferentiation. We were not able to determine so far if this is due to the loss of the plasmid or silencing. In tracking cell fate during regeneration, labeling a single cell is crucial. However, delivering the plasmid to a single cell by electroporation always leaves the possibility of inadvertent labeling adjacent cells. Furthermore, even when cell labeling is marked, the label is not stable. For example, the fate of muscle fibers in the regenerating tail is still an open question. The possibility of satellite cells populating the blastema and reconstituting muscle fibers of the tail not cells originating form the dedifferentiation of the muscle fiber still remains to be elucidated.

The triploid/diploid marker system has another disadvantage. The visualization of nucleoli requires fixing, sectioning and staining with bismuth (cite chicken and quail paper) therefore this method also limits *in vivo* long-term observations of blastema and regeneration.

The axolotl is a very suitable system for studying embryo development due to its very large embryo which is easily accessible for observations. The 2mm diameter of the embryo allows fair fidelity and reproducibility of transplantations. Previously Spemann and Mangold used salamanders to discover the organizing center of embryonic induction in 1924 (Spemann, 1924). However, technical difficulties pushed scientists toward easier system – *Xenopus*, where seasonal variability and accessibility of embryos is being overcome by hormone injections and *in vitro* fertilization. Availability of transgenic tools also played an important role in using the *Xenopus* system to study early development instead of axolotl.

The most effective way to overcome these problems and answer the above questions is to create transgenic animals which would allow the stable labeling of the cells or tissues.

Due to the complications of applying the *Xenopus* protocol to caudate eggs, described by Ueda by colleagues (Ueda et al., 2005), we chose to test transgenesis protocols that are widely used in teleosts. Transgenesis in teleosts is normally achieved via direct injection of plasmid DNA into the fertilized egg. This results in F0 animals expressing the transgene in a mosaic fashion, a proportion of which display germline transmission of the transgene (Culp et al., 1991; Stuart et al., 1988; Stuart et al., 1990). It was recently reported that coinjection of *ISceI* meganuclease with plasmids bearing its recognition sites enhances expression, decreases mosaicism in the F0 generation, and increases the germline transmission rate (Thermes et al., 2002). Here we describe plasmid injection into the one cell stage axolotl embryo as a method of generating transgenic animals and the evaluation of *ISceI* meganuclease's ability to enhance expression. Using this technique we report for the first time in the axolotl germline transmission of a transgene in a Mendelian ratio.

2.2 Estimation of the quality of embryos.

There are a few important issues to solve for in order for axolotl to become easily accessible and suitable for transgenesis. In the system of *Xenopus* there are well-established protocols of collecting embryos. Hormone injection is used to allow constant rather than seasonal production of large amount of eggs. Moreover, a very efficient protocol for embryo dejelling exists. Similar techniques have yet to be established for the axolotl.

The seasonal variability of embryos can be diminished to a great extent by breading animals in stable conditions all year long, which we can provide in our colony. So far we were not able to work out an efficient protocol for the collection of oocytes after hormone induction. From our experience so far, hormone injection in order to induce an animal to lay oocytes causes an unexplained imbalance in the animal's biology resulting in infertility. Because of this we were unable to work out the *in vitro* fertilization protocol.

Axolotl spawning results in 400-600 embryos on average. Under normal conditions, embryos are laid during the whole day and they are covered by protective eggshell and jelly coat. When the embryos are collected the spectrum of developmental stages varies from stage 1 to at least 6. The time necessary to complete the first cell division in axolotl is 6 hours. This gives an opportunity to collect a relatively large number of embryos in the one cell stage. In the *Xenopus* system there is an efficient protocol for dejelling embryos. It takes only 2 minutes to remove a *Xenopus* embryo coat by washing in 2% cysteine solution. Transgenesis by plasmid injection requires delivery of the transgene to the embryo at the earliest possible stage, before the first cell cleavage has taken place. This increases the probability of transgene integration into one set of the genome, and Mendelian transfer of the gene upon mating.

Here the average quality of embryos collected in our colony and the possibilities of efficient chemical dejelling in comparison to manual jelly coat removal is being investigated. The average quality of embryos in our colony was established by comparison of survival rates of 4 spawnings (Figure 1). Embryos were collected, counted and left to develop at room temperature; water was changed every second day, and dead embryos were removed. After two weeks embryos were scored again. 3

out of 4 batches showed survival rate of 65% of average, whereas a fourth batch showed only a 28% survival rate. This suggests that occasionally the ability of embryos to survive is low from the start and when used in an experiment may not deliver a conclusive or representative result. This observation was further confirmed during later experiments, where animals occasionally were dying for no apparent reason. On average, approximately 60% of embryos develop normally and survive until at least until stage 43, when the embryo naturally hatches out of the coat.

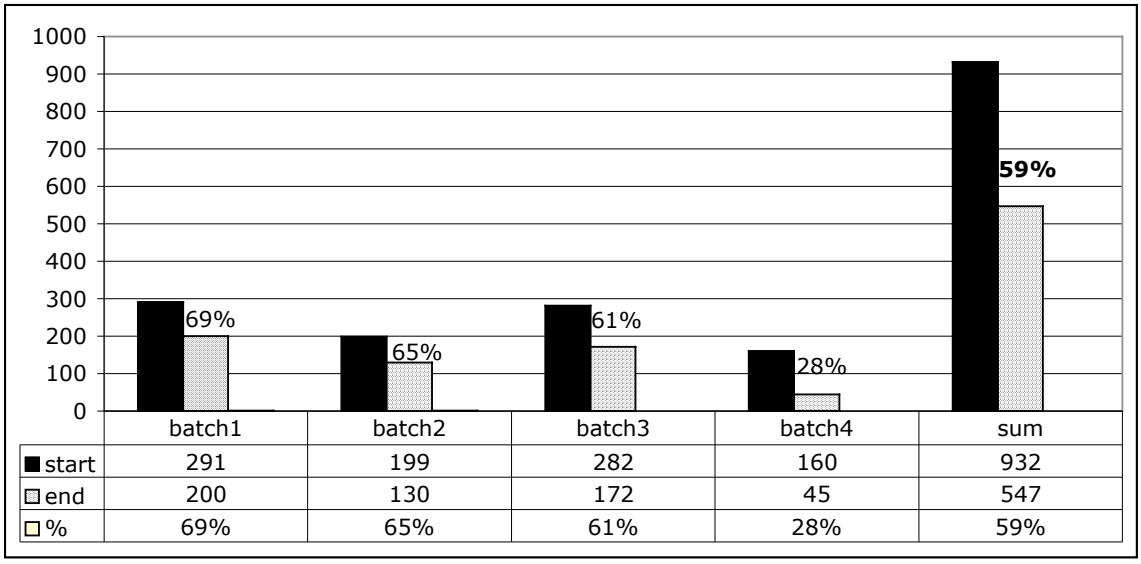
Knowing the average survival rate of embryos, it became necessary to test whether manual dejelling is damaging to the embryos. Several batches of embryos were dejellied and left to develop. In Figure 2 the comparison of two manually dejellied embryo batches. Batch No.1 represents a very good quality pool of embryos. In this situation manual dejelling has no effect on embryos' survival (and development). Therefore, careful manual procedure in sterile conditions is sufficient to obtain a large amount of embryos of the desired stage without mechanical damage. However, batch No.2 represents pool of bad quality embryos, which can be seen by the fact that even non-dejelied embryos survive poorly. In this case, mechanical removal of the coat is another factor contributing to the dramatic decrease of survival, and animals which do develop show severe malformations.

The 60% of surviving embryos and efficiency of manual dejelling was used as the base for establishing whether it is feasible to develop an efficient chemical dejelling protocol in axolotl, which would be comparable to the *Xenopus* protocol. The requirement was shortening the time of the dejelling procedure and maximum viability of embryos after treatment – which ideally is a 55-60% survival rate – close to the natural survival rate.

We chose several chemicals to test: 2% cysteine – very successfully used in *Xenopus*, collagenase (concentration range: 0.5mg/ml – 2mg/ml), dispase, collagenase/dispase mix, trypsin and 2.5% thioglycolic acid, pH=9, and various other combinations of these chemicals. Using 2.5% Thioglycolic acid, pH=9.0, with the time-range tested – 25-50 min, on average 70% embryos loose their coat, however, less than 10% survive longer than one week. In the case of collagenase, we used a concentration range of 0.5mg/ml – 2mg/ml within time points of 20 and 47 min. The coat removal was 60 and 86%, respectively, and survival of the embryos was less than 20% after 1 week. In the case of collagenase – dispase mix, in the concentrations of 2mg/ml/0.5mg/ml

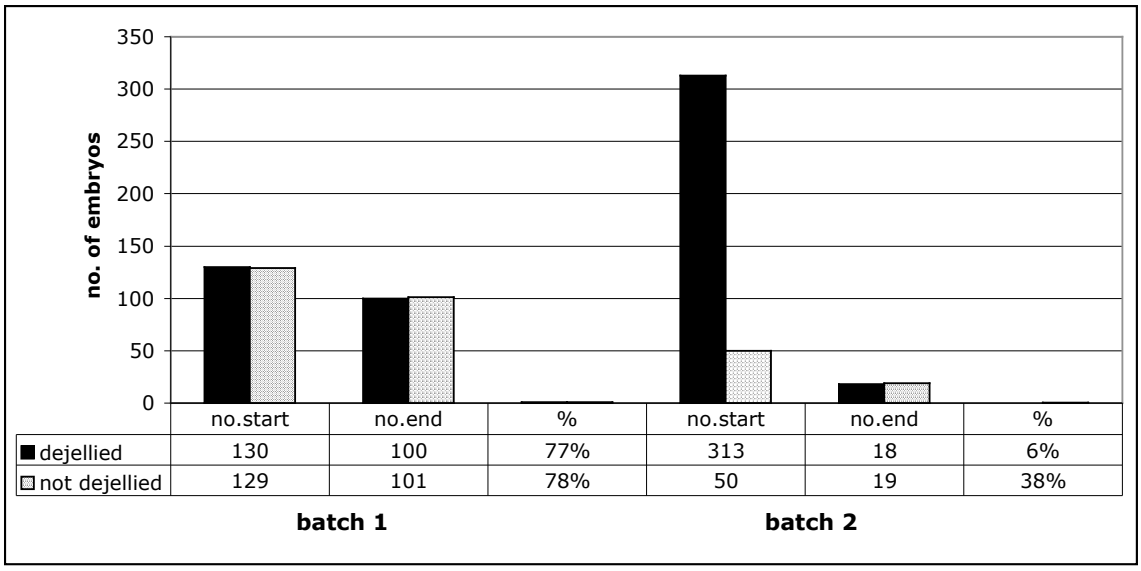
for 8 min the result was 57% coat removal and 2% survival after one week. Using the lower concentration of collagenase-dispase mix (0.5mg/ml;0.1mg/ml) for 47 min resulted in the removal of the eggshell in 83% of embryos with 43 % of them surviving one week. Dispase and trypsin alone even in low concentrations was very toxic to the embryos. Several combinations of proteases and 2.5%TA, pH=9, gave no satisfactory result in terms of timing and embryo survival. In the case of 2% cysteine treatment after 30 minutes, we observe only jelly coat removal and the eggshell remains intact.

The low concentration of collagenase-dispase mix (0.5mg/ml:0.1mg/ml) was the closest result to the relatively good survival observed normally. However, the duration of dejelling in comparison to *Xenopus*, where it takes 2 minutes to remove the coat, is unacceptable. No result lower than 10 minutes was obtained that gave coat removal in more than 90% of embryos and a survival rate higher than 60 % was reached. Therefore, it became necessary to master manual dejelling and this procedure was used to remove the coat from embryos in further experiments.



	batch1	batch2	batch3	batch4	sum
start	291	199	282	160	932
end	200	130	172	45	547
%	69%	65%	61%	28%	59%

Figure 1. Four batches of embryos were collected, scored and allowed to develop. Three of them have similar rates of survival after 1 month. Occasionally, a very poor quality batch occurs. On average, the survival rate of embryos is 60%.



	batch 1			batch 2		
	no.start	no.end	%	no.start	no.end	%
dejellied	130	100	77%	313	18	6%
not dejellied	129	101	78%	50	19	38%

Figure 2. Batches of embryos can have a very different quality. Here the effect of mechanical damage on healthy and unhealthy embryos during manual dejelling is shown. If spawning results in healthy embryos, careful manual dejelling has no effect on survival and development of embryos (batch1). However, the mechanical removal of the jelly coat from embryos more delicate, or less healthy, caused dramatic decrease in survival and severe developmental malformations.

2.3 Plasmid injection results in mosaic transgene expression with the duration of expression being promoter dependent

To assess the efficacy of plasmid injection for generating stable transgene expression, we tested plasmids harboring three different constitutive promoters driving green fluorescent protein: cytomegalovirus (CMVGFP), *X borealis* cytoskeletal actin (CSKAhGFP; (Thermes et al., 2002), and cytoplasmic actin fused to the CMV enhancer, (CAGGsEGFP; (Niwa et al., 1991). In this experiment we injected 2ng of DNA/egg. All plasmids yielded mosaic expression of fluorescent protein in the developing embryos ranging from the labeling of an occasional cell, to animals where at least 50% of the body cells expressed the transgene.

To evaluate and compare the extent of mosaicism and intensity of expression we categorized the hatchlings into four expression categories (Table 1). “Very strong” indicated animals that displayed almost ubiquitous expression of GFP so that under the stereomicroscope the intensity of fluorescence was relatively uniform and distinct patches of cells were not visible. The “strong” category denoted animals where expression was widespread and strong, but some mosaicism was evident, with clearly visible patches of muscle, skin, heart, blood cells, veins, neural system and notochord. “Moderate” denoted animals with a weaker intensity of fluorescence in greater than 50% of the cells or animals with a higher intensity of fluorescence in slightly less than 50% of cells. Such animals typically expressed the reporter gene mostly in body muscles and skin and with very few blood cells and heart labeled. The “weak” category represented weak expression of reporter gene and was mostly limited to small patches of body muscles and scattered patches of skin. From these observations it was clear that the intensity and extent of expression was promoter dependent (Table 1).

			Categories of expression at hatchling				*Duration and intensity of EGFP expression in time*		
Promoter	Survived/ Injected (%)	Rate of expression %(No)	Very strong No/% survivors	Strong No/% survivors	Moderate No/% survivors	Weak No/% survivors	Embryo	Hatchling	Larvae (2mth)
CMV	113/639 18%	34% (38)				38(100)	++	+	-
CSKA	10/180 6%	50% (5)			2(40)	3(60)	++	+	+
CAGGs	6/51 12%	100%(6)	1(17)	5(83)			+++	+++	+++

Table 1. Comparison of expression levels, duration of expression and survival rate of animals injected with three different promoters: CMV – cytomegalovirus promoter, CSKA – *X. borealis* cytoskeletal actin promoter, CAGGs – chicken b-actin promoter combined with IE CMV enhancer.

Significantly, the different promoters showed widely different persistence of expression over the course of development. The expression driven by the CMV promoter decreased steeply over time to the stage where all animals lost most of the fluorescent labeling by 2 months and only small, scattered groups of muscle or skin cells continued to express label (Table 1). The CSKA promoter, although a cellular promoter, was also mildly silenced over time. In contrast, the expression obtained from the CAGGs promoter was strong and most importantly this strong expression persisted beyond 6 months (Figure 3A and B). The one F0 male that displayed "very strong" GFP expression in close to all cells of the body was grown for 14 months to sexual maturity. Expression of GFP was stable throughout development and into adulthood (Figure 3A and B). Considering the persistence of the CAGGs-driven expression, it is likely that the CMV-driven construct is silenced in the genome over time. Indeed delivery of CMV promoter driven constructs into rat muscle via adenovirus injection is extensively silenced soon after delivery to the cell (Brooks et al., 2004). Silencing of CMV-promoter driven gene expression has also been described in transgenic Xenopus (Ryffel et al., 2003). In contrast the CAGGs promoter has been used for widespread expression in transgenic mice (for example: (Okabe et al., 1997), and our results confirm its effectiveness in promoting ubiquitous expression in transgenic animals.

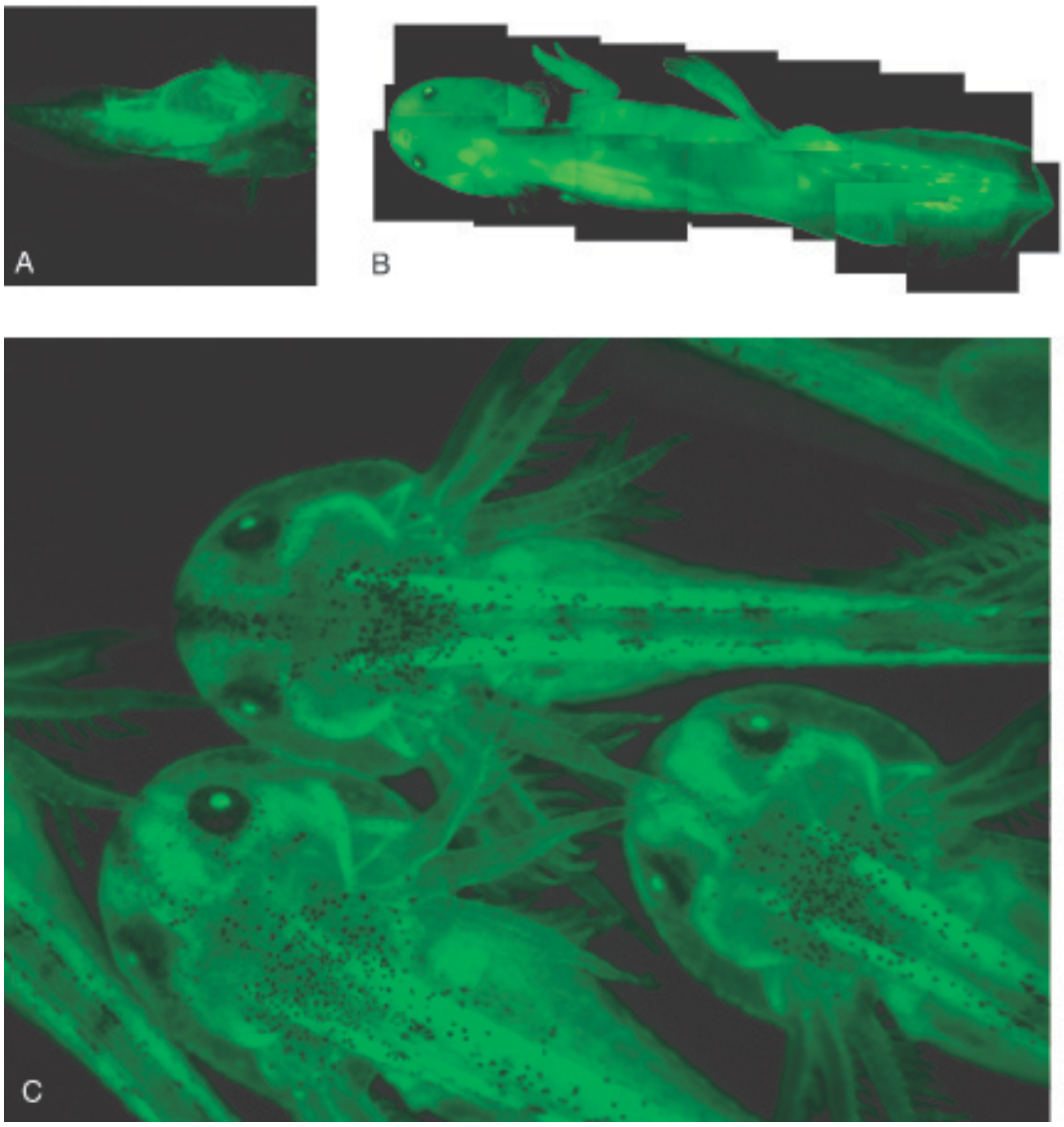


Figure 3. Axolotl injected with CAGGsEGFP construct - founder animal of our first transgenic line of axolotl. CAGGs - chicken B-actin+IE CMV promoter. A. Expression of EGFP after 1 month. The animal is about 1.2 cm long. B. Expression of EGFP in the same animal after 6 months. The animal is about 13 cm long. Expression at this level was observed until the first mating. C. F1 animals derived from mating the GFP+ of transgenic axolotl. Progeny are at the 2cm long larval stage. GFP is ubiquitously expressed all over the body.

2.4 Testing Adeno-associated virus inverted terminal repeats (AAV-ITRs) in the axolotl system

Fu and colleagues (Fu et al., 1998) have reported a strategy for efficient and stable expression of transgenes driven by both ubiquitous and tissue-specific promoters by direct DNA injection into developing *Xenopus* embryos. This strategy involves flanking expression cassettes of interest with inverted terminal repeat sequences (ITRs) from adeno-associated virus. Their results show that, when injected with CMVnBgal-ITRs (linearized), 65% of scored embryos expressed the transgene in more than 50% of the progeny of injected cells. Although these results look promising, they report that the amount of plasmid DNA in injected embryos decreases steadily from stage 22 onward, and at comparable rates for both ITR-containing and non-ITR-containing plasmids (southern blot analyses on DNA extracted from embryos in different stages), so the conclusion was that integration may be occurring at a relatively low frequency.

Moreover, the authors found that ITR sequences restrict ectopic expression of □-cardiac actin promoter. Whereas 52% of scored embryos injected with linearized control plasmid (no ITRs) exhibited ectopic GFP mRNA expression, only 19% of scored embryos injected with linearized ITR-containig plasmid exhibited ectopic GFP expression and in the latter group ectopic expression was limited to one or very few cells. Considering both the resemblance of *Xenopus* and axolotl and the ability of AAV ITRs to function both in mammalian cells and in *Xenopus*, it is reasonable to think that this method may give similar efficiencies in our system within a single generation.

In vivo studies on axolotl tail regeneration require the use of an easily detectable and traceable marker. Therefore, GFP was used instead of nβgal (see Materials and Methods). Initially the 0.01-1ug/ul range of plasmid concentration in the injection solution was tested, resulting in the best survival with the lowest concentration (Table 2). However, the expression in surviving animals was so weak that we decided to further use 0.1ug/μl in 20nl drop per embryo, sacrificing the best survival for a better expression.

pCS2 GFP ITR	*C (ug/ul)*	*Nr of injected embryos*	*Nr of survived embryos*	*% of survived embryos*
	0.01	40	17	43%
	0.1	40	4	10%
	1	40	0	0

Table 2. Titration of pCS2GFPITR plasmid in order to test embryo survival. It is dependant on the plasmid concentration in the injection solution. Embryos were scored 3 weeks after injection.

Further experiments were designed to compare expression of GFP obtained from a plasmid containing ITRs (in circular and linear version) with the same plasmid without ITRs. The goal of this experiment was to confirm if it is possible using the *Xenopus* protocol to reach similar efficiencies in the axolotl system and if it is possible to apply this method to obtain transgenic axolotl lines. Table 3 shows the result of injecting a plasmid containing ITRs compared to non-ITR plasmid injection.

	Nr of Injected embryos	*Nr of Survived embryos*	*% of Survived embryos*	*Nr of Survivors expressing GFP*	*% of Survivors expressing GFP*
pCS2GFPITR	400	54	14	19	35
pCS2GFPITR(lin)	80	14	18	8	57
pCS2GFP	78	17	22	-	-
pCS2GFP(lin)	79	23	29	-	-

Table 3. Evaluation of AAV-ITR transgenesis method in axolotl. Animals were scored for survival and the presence of GFP expression after 4 weeks. (lin) – plasmid linearized.

As we could observe, the survival of embryos does not exceed 30% with a slight decrease in survival when viral ITR sequences were included in the plasmid. Linearization of the plasmid shows a tendency for higher survival. In the case of plasmids without ITRs, none of the surviving animals expressed GFP at the time of examination (4 weeks). Only 35% of the survivors in the case of circular plasmid injection express GFP. A slightly higher number of animals expressing GFP is observed when a linear plasmid was injected. The expression was first examined under a fluorescent microscope after 4 weeks. The expression level was moderate and mosaic with a different intensity of labeled cells. Mostly muscle fibers were labeled,

with occasional patches of skin and blood vessels. Moreover, after 6 weeks, expression had gradually decreased to only a few muscle fibers labeled in most cases. No animal with strong, ubiquitous and stable expression of GFP was obtained with this method.

There are several explanations for this result. One possibility is that no integration occurred; DNA remained episomal and was distributed as such to descendant cells in the developing embryo. However, with a very low frequency, the possibility of integration of the transgene in later stages of development exists, as evidenced by some cells remaining GFP+ for more than 5 months. Another phenomenon possibly explaining the decrease of GFP expression could be the silencing of integrated CMV promoter (Garrick et al., 1998).

Having this result, it could not be confirmed that AAV-ITR act to promote or enhance integration of the transgene in the axolotl system. Animals produced using this method do not seem to have the potential for germline transmission and, since the expression decreases, they cannot be used for lineage tracing experiments. However, the short-term expression (during embryonic stages) was clearly higher than in the case of the non-ITR plasmid. Hence, it could be used for expression of transgenes for studying embryonic development. The tissue specific expression or non-viral universal promoter in combination with ITRs was not tested.

2.5 Coinjection of ISceI meganuclease enzyme with a plasmid containing its recognition sites enhances transgene expression

One goal was to develop a transgenesis method where we could produce relatively large numbers of animals with the least mosaicism possible in the F0 generation so that the F0 animals might be used directly in cell tracking experiments, as is done in *Xenopus*. A second consideration was germline transmission of the transgene. To reduce mosaicism in the F0 generation and to increase the chance of germline transmission we tested a method successfully implemented in medaka involving coinjection of *ISceI* enzyme mixed with plasmid containing its recognition sites (Thermes et al., 2002). *ISceI* meganuclease is an intron-encoded homing endonuclease with an 18 bp long recognition sites that was isolated from *S.cerevisiae* (Jacquier and Dujon, 1985). The meganuclease enzyme induces double stranded break formation at its recognition sites and probably also takes part in double strand break repair (Rouet et al., 1994).

To test whether the *ISceI* method alleviates mosaicism, we constructed a pCAGGs-EGFP plasmid where Sce sites flanked the expression cassette and injected the pCAGGsEGFP(Sce) and pCAGGsEGFP plasmids with and without enzyme. In initial experiments we tested optimal plasmid and enzyme concentrations by a series of plasmid injections in the range from 2ng to 500pg per embryo. Optimal results were obtained with injection of 1ng of plasmid per embryo. With this amount of plasmid the optimal enzyme amount was established by series of injections where the enzyme concentration was varied from 0.01 to 4U/µl. 1U/μl (10nU/embryo) of enzyme in the injection mix yielded optimal results in terms of embryo survival but most of all the number of animals showing very strong expression of GFP was highest. Co-injection of pCAGGsEGFP(Sce) with the *ISceI* enzyme resulted in 16% of animals classified as “very strong” (Figure 4) in comparison to the control injections where only 2% of animals were in this class (Table 4).

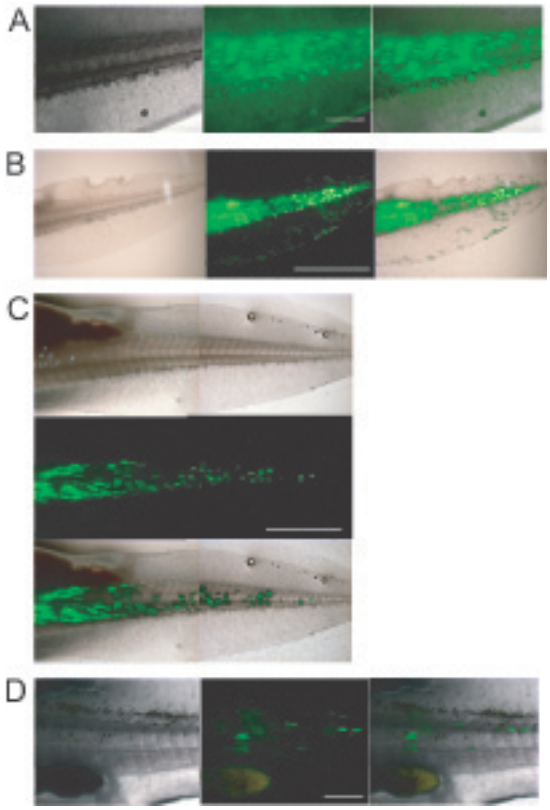


Figure 4. Animals obtained from co-injections of GFP plasmid and *ISceI* meganuclease enzyme were scored according to categories A**, very strong;** B**, strong;** C**, moderate;** D**, weak. Scale bars: A, D 0.1cm, B 0.5cm, C 0.3cm.**

Construct	*Injected No*	*Survived No/%*	*Very strong No/% survivors*	*Strong No/% survivors*	*Moderate No/% survivors*	*Weak No/% survivors*
CAGGS GFP SCE+E	281	92 (33%)	15 (16%)	9 (10%)	31 (34%)	37 (40%)
CAGGS GFP SCE	157	74 (47%)	0	8 (11%)	23 (31%)	43 (58%)
CAGGS GFP+E	291	68 (23%)	0	3 (4%)	9 (13%)	56 (82%)
CAGGS GFP	223	61 (27%)	1 (2%)	3 (5%)	14 (23%)	43 (70%)
E	155	103 (66%)				

Table 4. Embryos at one cell stage were injected with various combinations of plasmid and *I-SceI* enzyme. The quality of expression was checked after 1.5 months and animals were classified in categories of expression described in the text; E- *I-SceI* meganuclease enzyme.

2.6 Germline transmission of transgene expression

To evaluate germline transmission we raised 15 F0 animals harboring the pCAGGsGFP plasmid to adulthood and performed matings with the white mutant. This experiment was performed prior to obtaining the animals from the meganuclease experiments described above. Therefore, the animals used for mating represented one male with "very strong" expression, and 14 animals with "moderate or strong" expression (Figure 4). In such matings 6 out of 15 animals produced at least some GFP+ embryos in the F1 generation, with 3 animals generating over 20% GFP+ eggs (Figure 4 and Table 5).

Animal	*Sex (M/F)*	*No of EGFP+ embryos*	*No of EGFP- embryos*	*% embryos GFP+*
1 (very strong)	M	160	150	52
2	F	159	213	43
3	F	0	150	0
4	F	0	183	0
5	F	10	163	6
6	M	3	311	1
7	F	0	46	0
8	M	0	62	0
9	F	0	207	0
10	M	0	175	0
11	F	8	493	1
12	F	0	59	0
13	F	0	80	0
14	M	40	152	21
15	M	0	243	0

Table 5. Germline transmission of pCAGGsEGFP

Notably, the mating of the "very strong" male depicted in Figure 1 with a white female produced clutches where 50% of the eggs were GFP+. This result suggested that in this case, the transgene might be expressed from a single chromosome in all germ cells, resulting in Mendelian inheritance of the transgene. To confirm this ratio, this male was used for a second mating, resulting again in 50% eggs GFP+, supporting the notion of Mendelian inheritance of the transgene. In the eggs from these matings, GFP expression was first observed after the mid-blastula transition

(stage 8 of development) consistent with zygotic expression of the GFP transgene from the male chromosome.

Figure 3C depicts the GFP+ F1 transgenic animals at the larval stage. The animals appear to express GFP uniformly throughout their body. To examine in more detail whether the GFP transgene was ubiquitously expressed in the F1 animals various tissues from an F1 transgenic animal were compared to those from a white animal of the same age. Strong, ubiquitous GFP expression was observed in dissected forelimbs, eyes, hearts, lungs and livers (Figure 5). Due to our interest in tail regeneration and blastema formation we examined GFP expression at higher resolution in longitudinal cryosections of regenerating tails (Figure 6A-C). All tissues including mature and regenerating spinal cord (SC), the notochord (N), epidermis (E), and the blastema (B) were uniformly GFP+. By examining cells at high resolution in the tail fin (Figure 6D-F) and the tail blastema (Figure 6G-I) we could ascertain that all identifiable Höchst-positive cells were also GFP+. This is particularly clear in the dorsal tail fin where individual cells are widely separated from each other and embedded in a dense extracellular matrix (Figure 6D-F).

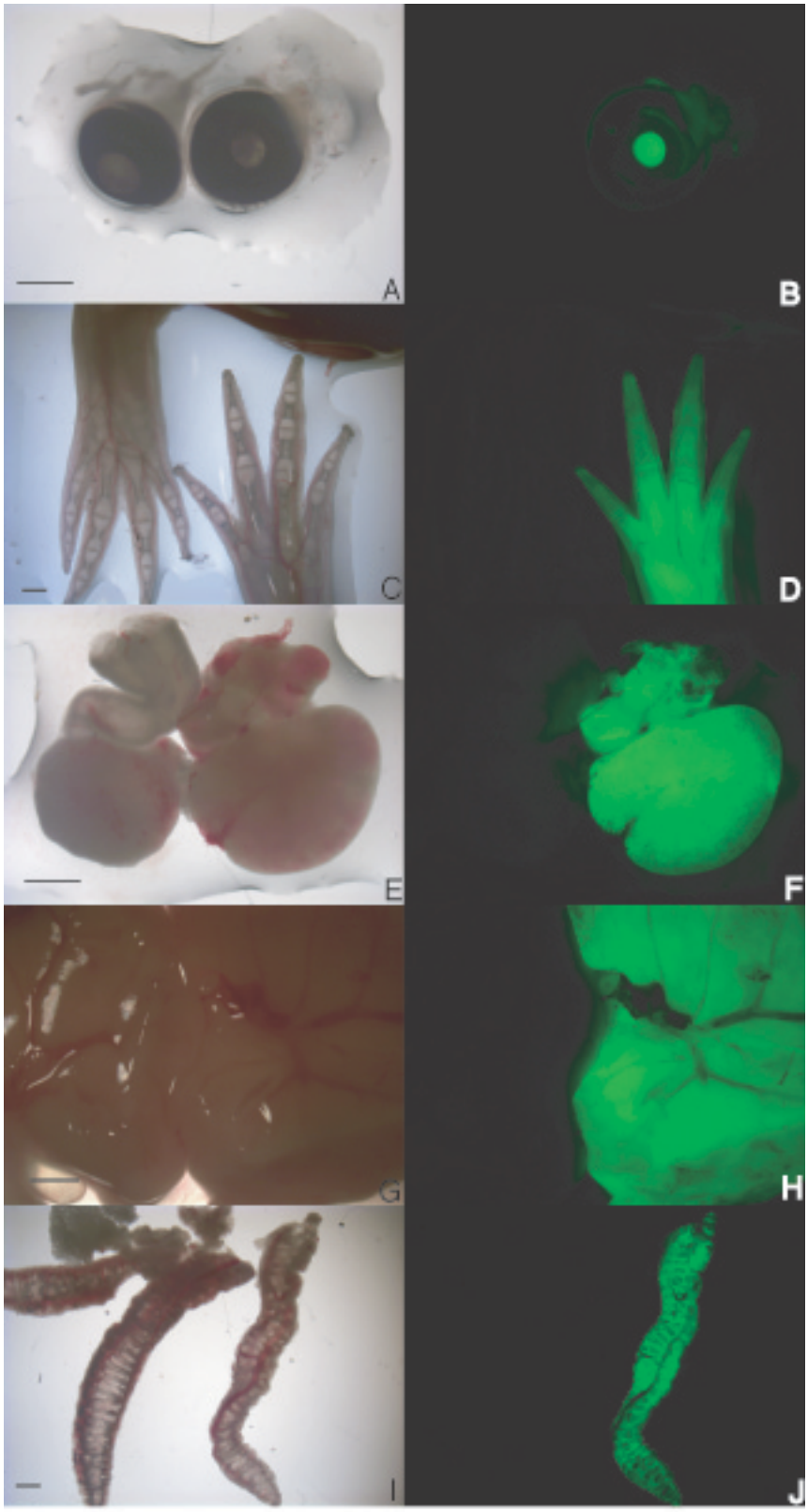


Figure 5. GFP fluorescence of major organs and stuctures in GFP-transgenic compared to a non-transgenic animal. A and B shows comparison of eyes, C,D forelimbs, E,F heart, G,H liver, I, J lung. On figures A, C, E, G, I brightfield and on B, D, F, H, J fluorescent images are shown. Scale bars - A, B, C, D, E, F, I, J - 1mm, G, H - 2mm.

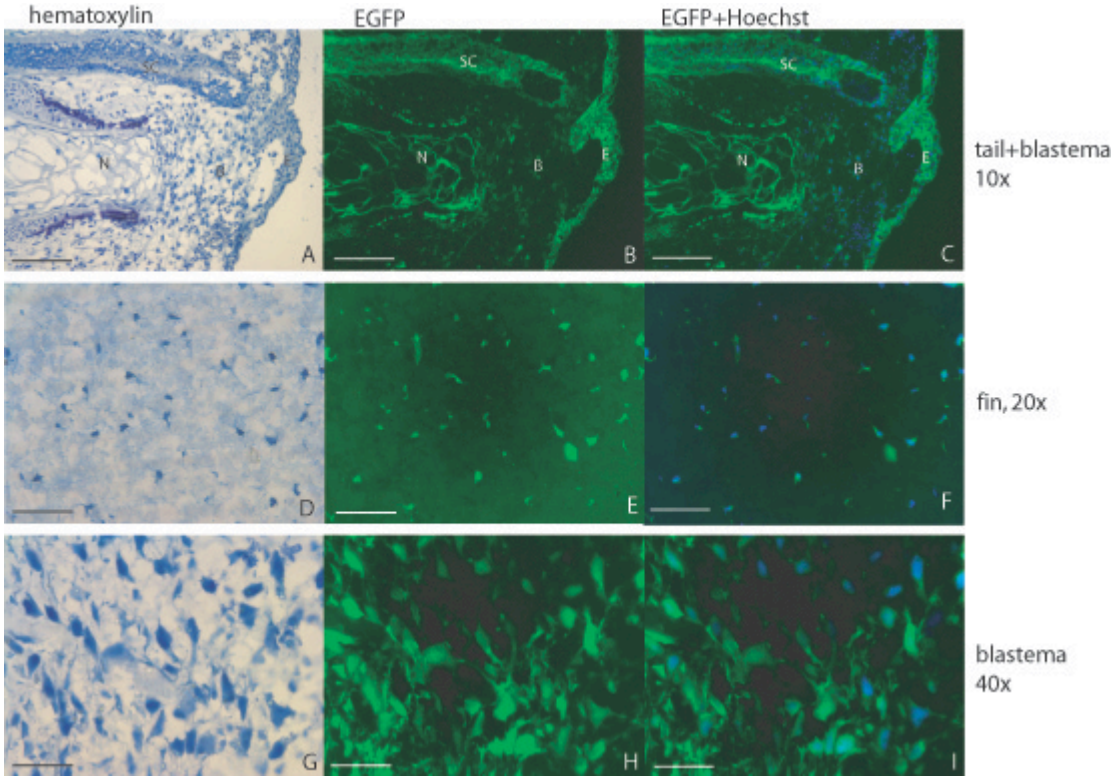


Figure 6. High resolution images of GFP fluorescence in cryosections of a regenerating axolotl tail. A regenerating tail of a transgenic fluorescent animal was sectioned longitudinally and stained with hematoxylin to visualize cells and Höchst to visualize nuclei. A, B, C An overview of the tail tissues. sc – spinal cord, n – notochord, b – blastema, e – epidermis. D, E, F Higher magnification view of tail fin cells. Note in hematoxylin and Höchst staning that cells are widely separated from each other via ECM. All cells are GFP+ . G,H,I, High magnification view of tail blastema cells. All Höchst positive cells are GFP+. A,D,G, hematoxylin staining. B,E,H, GFP fluorescence from transgenic animal, C,F,I overlay of GFP and Hoechst staining. Scale bars: A-C 200μm; D-F 100μm; G-I 50μm.

2.7 Somite and neural crest contribute to dorsal fin mesenchyme

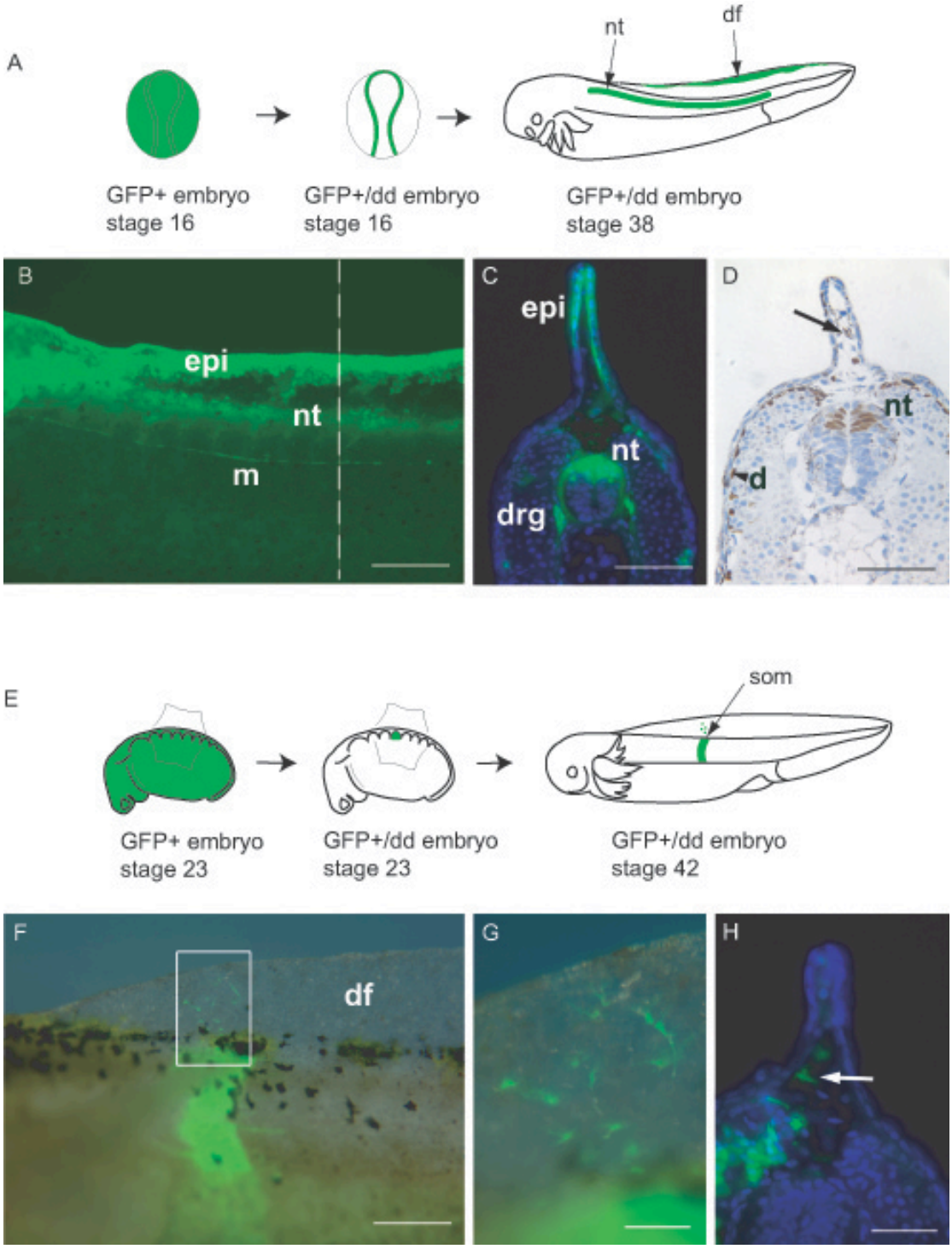
An animal constitutively expressing GFP is a valuable tool for analyzing the fate of cells during development and regeneration by grafting GFP+ cells into unlabeled hosts. The axolotl is particularly amenable to such an approach as tissues at all stages (embryonic, larval, juvenile and adult) engraft from one animal to another extremely well with no evidence of rejection. Here we have used grafting of GFP+ tissues to study cell fate during embryogenesis and regeneration. We have focused on the formation of the dorsal fin during embryogenesis, and the contribution of blood-derived cells to tail regeneration.

We were interested in the genesis and composition of the dorsal fin mesenchyme since it is a structure that contributes to tail regeneration but has been little studied. The fin mesenchyme is composed of stellate cells embedded in a dense extracellular matrix (Figure 6 D,E,F). In lower vertebrates the mesenchyme of the dorsal fin has been classically assumed to be completely neural crest-derived (Du Shane, 1935; Raven, 1931), for schematic see (Tucker and Slack, 2004). To confirm the neural crest origin of dorsal fin mesenchyme, we replaced trunk neural folds (8 cases) and cranial folds in addition to trunk neural folds (2 cases) with GFP+ tissue at stage 16 and examined whether the dorsal fin was homogeneously labeled (Figure 7A). Surprisingly only the apical portions of the dorsal fin epidermis (stage 40-41) were GFP+ in all larvae (Figure 7B). Transverse vibratome sections (4 individuals) showed that only half of the internal fin mesenchyme cells were GFP+ and also demonstrated the complete and specific labeling of the neural crest (Figure 7C). These results suggested a possible additional non-neural crest contribution to the dorsal fin mesenchyme.

A hint to a potential additional source of dorsal fin mesenchyme came from PAX7 immunohistochemistry of stage 39-40 animals. In addition to the expected immunostaining in the dorsal neural tube (but not in the neural crest nor neural crest derived structures such as dorsal root ganglia), PAX7+ cells were observed in the dermatome (Figure 7D) as well as some cells in the dorsal fin mesenchyme (Figure

7D). This raised the possibility that the dermatome may contribute mesenchyme to the dorsal fin. To test this, we grafted GFP+ somites into unlabeled hosts at stage 23, well before neural crest has delaminated and migrated from the neural tube (Figure 7E). Six out of seven larvae (stage 42) displayed GFP+ cells in the dorsal fin that sometimes populated even the apical-most regions of the fin (Figure 7F, G). Transverse sections revealed that labeled cells were localized within the mesenchyme (Figure 7H). As expected, no GFP+ neural crest derivatives were found in such grafted animals, confirming the specificity of the transplant. These experiments indicate that dorsal fin mesenchyme derives not only from neural crest but also significantly from the dermatome of the somites.

Figure 7. Dual origin of dorsal fin mesenchyme. A**. Schematic showing grafting of GFP+ neural folds to unlabelled hosts in order to investigate the contribution of neural crest to the dorsal fin. Both cranial and trunk neural folds were labeled.** B**. Left side of a white axolotl larva (head to the left, outside the image; stage 40-41) in which both cranial and trunk neural folds had been replaced with GFP+ neural folds as depicted in** A**. The label is visible in the apex of the dorsal fin epidermis (epi), the neural tube (nt) and the middle lateral line nerve (m). Dotted line denotes the level of transverse section shown in** C**.** C. **Transverse vibratome section through the larva shown in** B **demonstrates labeling of the apical part of the dorsal fin epidermis (epi). Fin mesenchyme is only sparsely labeled. Higher magnification views showed approximately 50% of mesenchymal cells labeled (data not shown). Dorsal neural tube (nt) and dorsal root ganglia (drg) are stained.** D. **Anti-PAX7 immunostaining of a transverse paraffin section through the trunk of a larva at stage 39-40. Cells in the dorsal neural tube (nt), dermatome (d) and dorsal fin mesenchyme (arrow) reacted positively.** E. **Grafting of one, left, GFP+ mid-trunk somite to unlabelled host in order to investigate possible contribution of dermatome cells to dorsal fin mesenchyme.** F. **Left side of a white axolotl larva (head to the left, outside the image; stage 40-41) in which one somite had been replaced with a GFP+ somite as described in** E**. GFP+ cells, very likely dermatome cells, have left the somite and havew migrated into the dorsal fin (df; enlargement see** G**).** H. **Transverse vibratome section through GFP+ somite revealing migrated GFP+ cells (arrows) within the dorsal fin mesenchyme. Scale bars: B, 250 μm; C,D,F,H, 100 μm; G, 50 μm.**


A
nt
df
GFP+ embryo
stage 16
GFP+/dd embryo
stage 16
GFP+/dd embryo
stage 38
B
epi
nt
m
C
epi
nt
drg
D
nt
d
E
som
GFP+ embryo
stage 23
GFP+/dd embryo
stage 23
GFP+/dd embryo
stage 42
F
df
G
H

2.8 The contribution of blood cells to tail regeneration.

In the following experiments, we have used embryonic transplantation of the blood anlage in order to label blood in the developing axolotl larvae, in order to track the fate of blood cells during tail regeneration. At stage 18, we transplanted posterior ventrolateral mesodermal areas (with overlying epidermis) that will form the ventral blood islands (Figure 8A) (Yamada, 1938). In urodeles, this region appears to be the major source of primitive and definitive hematopoiesis (Deparis and Jaylet, 1984) (Durand et al., 2002). Of 30 transplants, 3 animals survived to 2 cm long larval stages where we could follow the course of tail regeneration. To estimate the percentage of circulating blood that derived from the GFP graft, we acquired blood samples from the fully GFP+ germline transgenic animals and compared it to blood from one of the grafted animals (Figure 9). In the fully transgenic animals, the erythrocytes were not GFP+ while the non-erythrocytic white blood cells were GFP+ resulting in 3.2% of the circulating blood cells being GFP+ in the fully transgenic animals. When we examined the blood from one of the grafted animals, 1% of the circulating blood was GFP+ indicating that we had labeled a significant proportion of the blood-forming region. It is important to note here, that axolotl erythrocytes, despite of having a nucleus, don't express GFP, what was confirmed by Western blot analysis. This is due to transcriptional repression in these cells (Walmsley et al., 1991). In the grafted animals, we could observe GFP+ cells in circulating blood, as well as GFP+ cells that were evenly distributed throughout the tail including the dorsal fin (Figure 8B and C). The primary cell type that we observed labeled were Langerhans cells, identified based on morphology, which are dendritic cells primarily found in skin, dermis and lymph nodes. Figure 8D and E show Langerhans cells with their characteristic cell extensions. The occurrence of GFP+ cells containing melanin granules inside their cytoplasm (Figure 8E) confirms that these are Langerhans cells that are processing skin antigens (for review see: (Romani et al., 2003). We also observed other cells with a smaller, rounded to polygonal shape, that are presumably macrophages and leukocytes (Figure 8F) (Jones and Corwin, 1996). The presence of Langerhans cells and macrophages confirms that our transplants label myeloid lineages.

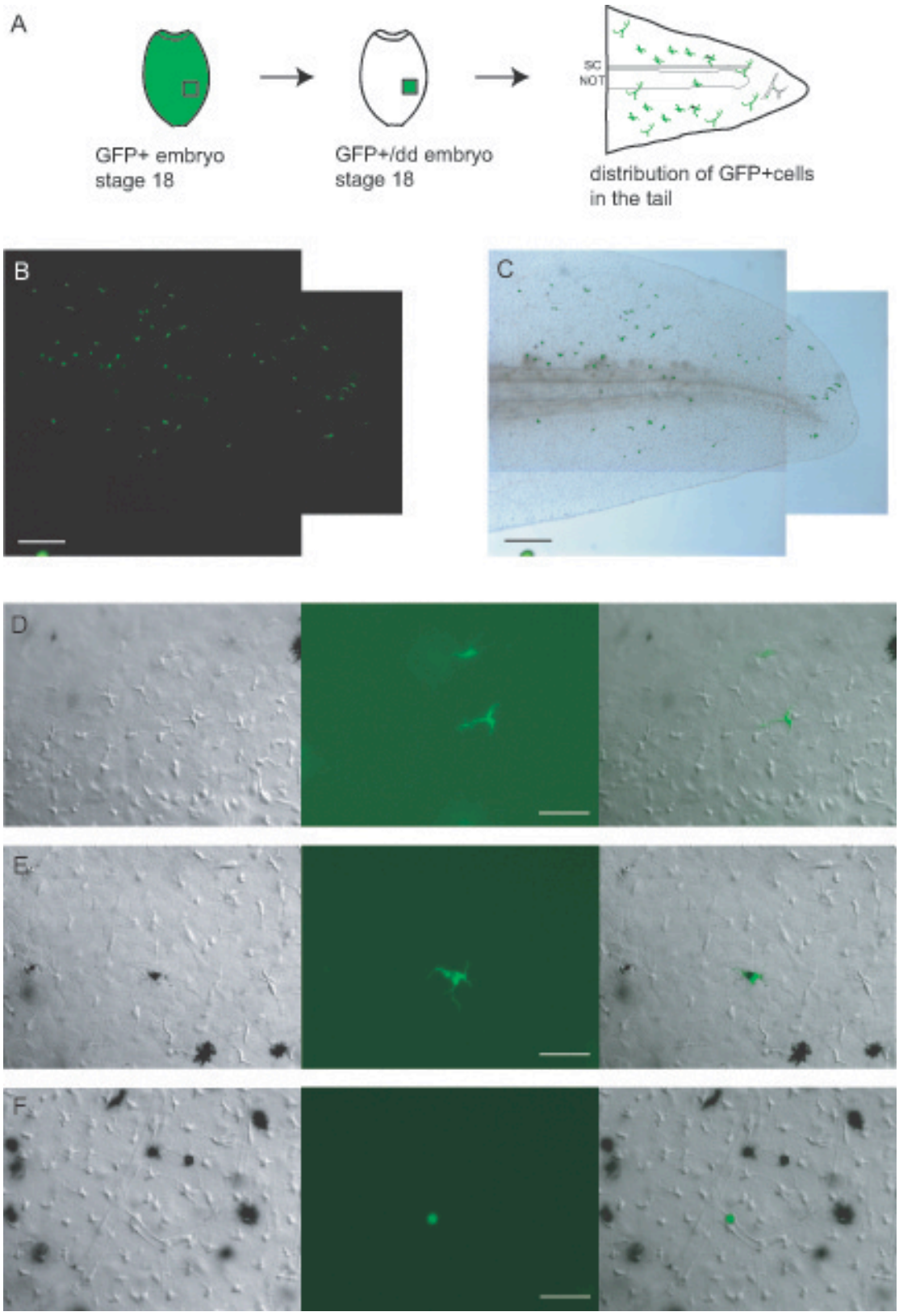

A
GFP+ embryo
stage 18
GFP+/dd embryo
stage 18
SC
NOT
distribution of GFP+cells
in the tail
B
C
D
E
F

Figure 8. Identification of GFP+ blood cell types in the mature tail after grafting GFP+ blood anlage to unlabelled hosts. A. Schematic showing grafting of GFP+ ventro-lateral tissue at stage 18 to unlabelled hosts and the subsequent pattern of labeling observed in the tail. sc – spinal cord, not – notochord. B. Fluorescent image of a tail derived from grafting experiments. C. Overlay of fluorescence and bright field image. D. High magnification images of a Langerhans cell in the dorsal fin; DIC, fluorescence and overlaid images. E. High magnification images of a Langerhans cell in the dorsal fin that has taken up melanin granules; DIC, fluorescence and overlayed images. F. A second subtype of GFP+ cell that likely represents a macrophage; DIC, fluorescence and overlaid images. Scale bars: B, C 0.5mm; D, E, F 50μm.

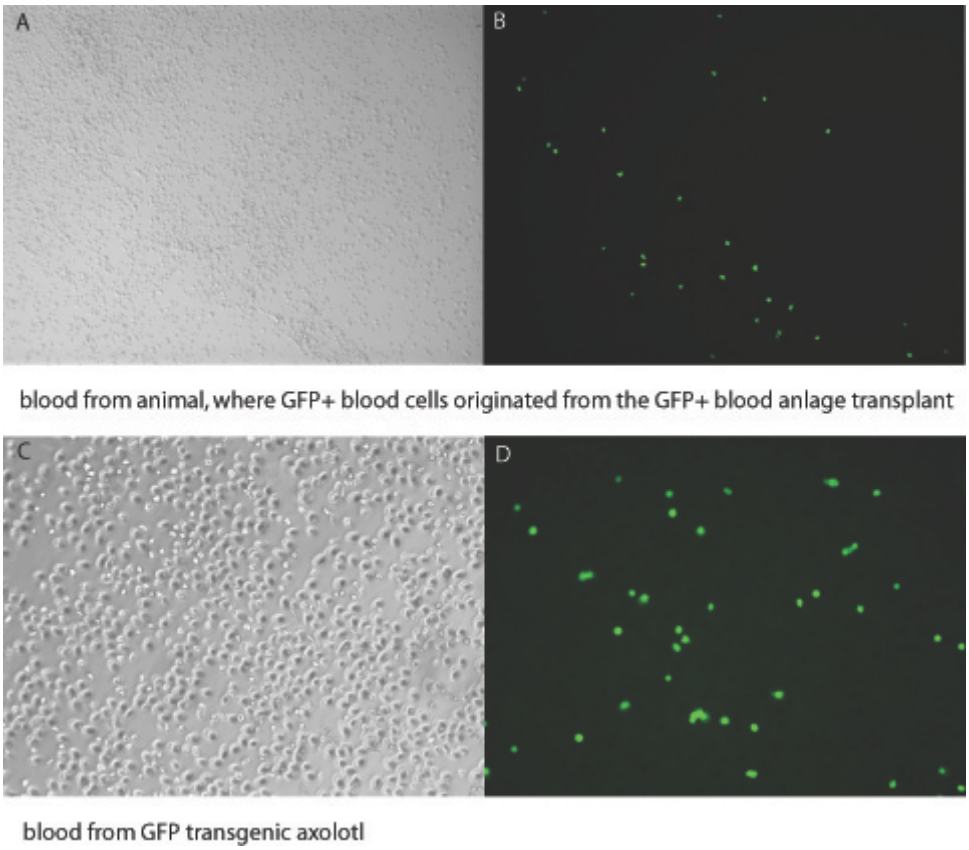


Figure 9. Distribution of GFP+ cells in the blood from an animal with transplanted blood forming region compared to blood of GFP+ transgenic axolotl. Blood was collected from the tail and the heart. A and B show the low magnification DIC and fluorescent image, respectively, of the blood of the experimental animal. C and D represent the DIC and fluorescent image of the distribution of GFP+ cells in the blood from GFP+ transgenic axolotl.

To determine the contribution of blood cells to regenerating tissues we amputated the grafted animals when they were 2 cm long larvae and allowed the tail to regenerate

for 2 weeks (Figure 10A). Each animal was amputated twice, bringing the total number of observations to six. Tail amputation caused rapid blood efflux, followed within several minutes by the formation of a blood clot. After several hours the blood clot was enclosed by the wound epidermis and became part of the blastema. One day post-amputation we observed an accumulation of GFP+ cells close to the amputation plane, either as a result of blood clotting, or due to the migration of GFP+ cells towards the wound (Figure 10D). Analysis of the regenerated part of the tail within the first few days after the lesion shows that the GFP+ cells that appear in the regenerated tissue are similar in shape as those in the tail before the regeneration (Figure 11). After 13 days, we observed the same even distribution of stellate GFP+ cells in the tail, with no contribution to muscle, cartilage or spinal cord (Figure 10H, I). Transverse sections of the regenerated tail confirmed that the GFP+ cells were not located within muscle, cartilage or spinal cord tissue.

Due to the stability of the GFP marker in transplanted animals, we are able to track the fate of those cells over the course of development. In those transplanted animals, at the age of three months we observe a number of muscle fibers appearing in the non-amputated area of the tail. In all cases, muscle fibers appear in the close proximity of the amputation plane. However, it is unclear whether those cells originated from hematopoietic cells via transdifferentiation, or blood derived cells fused with muscle progenitor cell. Therefore, in this case, a long-term cell tracking needs to be preformed supported by histological analysis of the tissues of interest.

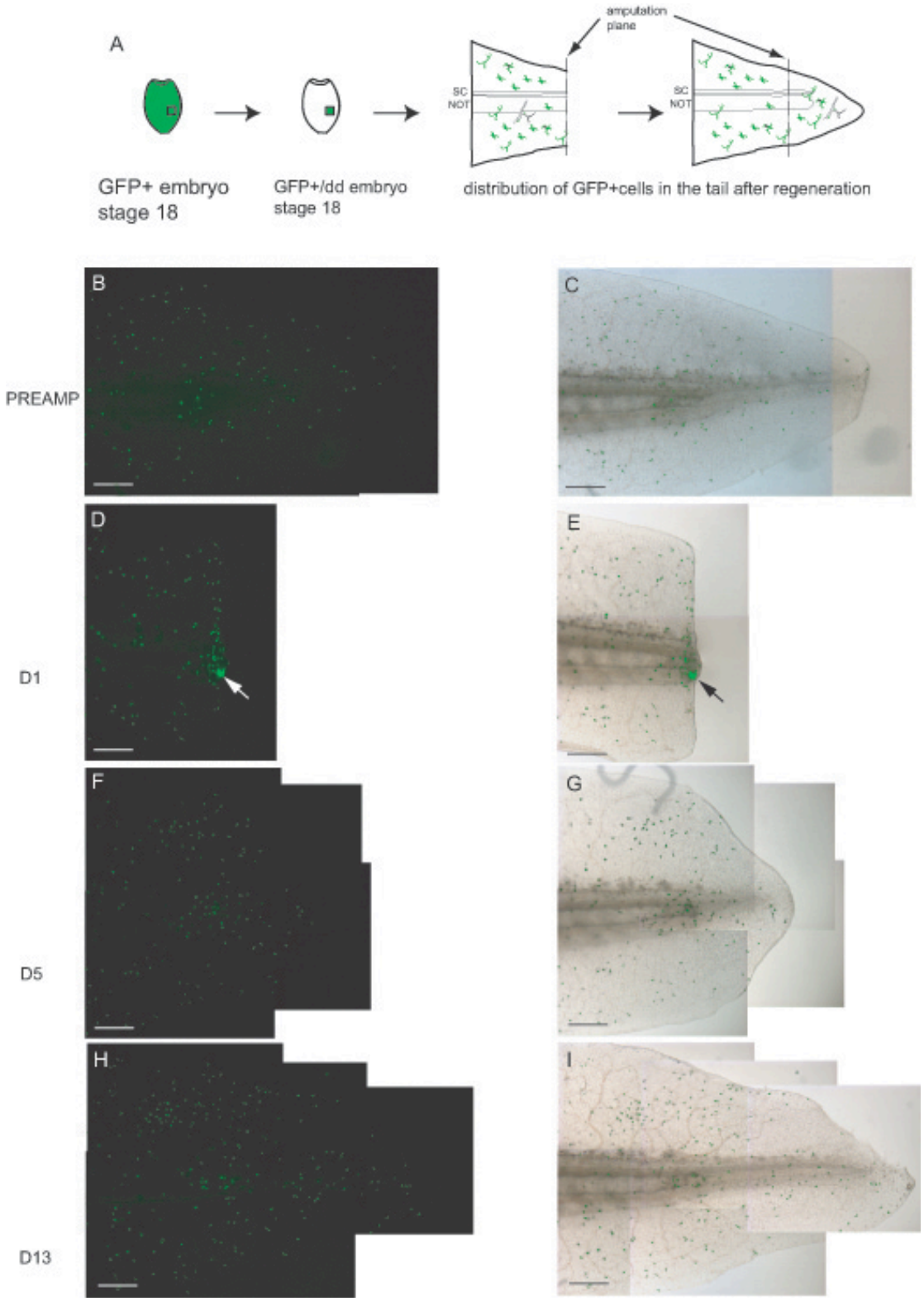

A
GFP+ embryo
stage 18
GFP+/dd embryo
stage 18
amputation
plane
SC
NOT
SC
NOT
distribution of GFP+cells in the tail after regeneration
PREAMP
D1
D5
D13
B
C
D
E
F
G
H
I

Figure 10. Tail amputation of transplanted animals containing GFP+ blood cells. A. Schematic showing grafting of GFP+ ventro-lateral tissue at stage 18 to unlabelled hosts and the subsequent pattern of labeling observed in the tail. After reaching a size of 1.5-2 cm animals were subjected to amputation and the contribution of GFP+ blood and its derivatives to the regenerated tail was followed over 13 days. sc – spinal cord, not – notochord. B and C Fluorescent and overlay images of the tail preamputation. D and E Fluorescent and overlay images respectively of the regenerated tail after one day. Arrow indicates group of cells embedded in the blood clot. F and G Regenerated tail after five days (fluorescence and overlay respectively). GFP+ cells have populated the growing blastema. H and I Almost fully regenerated tail after 13 days, where tail structures including spinal cord, cartilage and muscle have started differentiating. GFP+ cells populated the regenerated tail with a similar distribution as in the mature tissue. No labeling of muscle fibers, spinal cord or cartilage was observed. Scale bars: B – I 0.5mm.

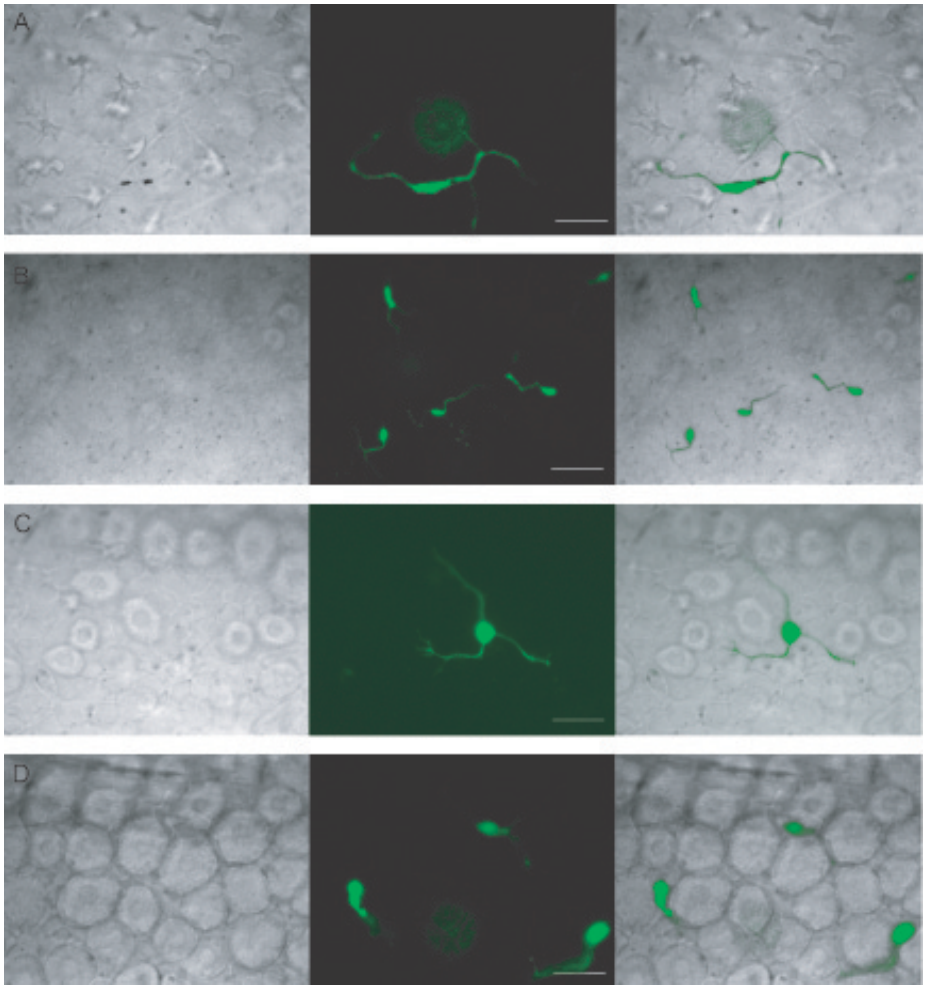


Figure 11. Representative spectrum of cells observed in the regenerated tail. Left, DIC; Middle, Fluorescence; Right, overlay. Scale bars, 25 μm.

2.9 Can blastema contribute to the spinal cord?

There are two notions of a blastema origin. One, postulates that stem cells residing in the tissue and their activation upon injury the main source of blastema structure (Cameron, 1986; Weiss, 1939). Second, discusses the phenomenon of dedifferentiation of mature cells as a source of blastema. Butler (Butler, 1942) formulated the theory of "local origin" of blastema, where dedifferentiation of mesodermal tissues at the amputation plane contributes to the blastema. The "model" was further observed in work of Hay (Hay, 1959; Hay and Fischman, 1961), where histological analysis showed DNA synthesis in muscle nuclei, loss of distinct muscle myofibrillar structure and presence of those cell within the blastema structure. Another source of blastema cells was shown by Muneoka and colleagues (Muneoka et al., 1986). Using diploid/triploid marker system they confirmed that dermis and cartilage contribute to the blastema. Moreover, most recent experiment done by Echeveri (Echeverri et al., 2001) points toward a dedifferentiation of a mature muscle fiber as a main contributor to the blastema.

If stem cells and dedifferentiation of mature tissues contributes to blastema, are those blastema cells equally pluripotent or the structure is a poll of heterogenous cells with distinct potentials (Figure 12)? This problem is still an open question in the field and only development of more sophisticated tools can help to answer those questions.

Tail regeneration in axolotl occurs through the phase of blastema formation. However, the spinal cord seems to reconstitute itself and already after 2 days the distinct shape of the terminal vesicle is visible. The spinal cord grows as a separate entity within the blastema environment and itself regenerates all cells withing the structure (Benraiss et al., 1999; Egar et al., 1970; Nordlander and Singer, 1978). What is incredibly interesting, the urodele amphibians are the only vertebrate that can regenerate fully functional spinal cord. In the axolotl, the spinal cord maintains neurogenic activity throughout the whole life of the animal constantly providing undifferentiated neural stem cells (Holder et al., 1991). There is evidence of ependymal cells residing in the lumen of the spinal cord switching their fate to other types of cells in the regenerating tail. The change in the identity goes as far as

switching germlayer lineage from ectoderm to mesoderm as it is in neural to muscle cell type transition (Echeverri and Tanaka, 2002a).

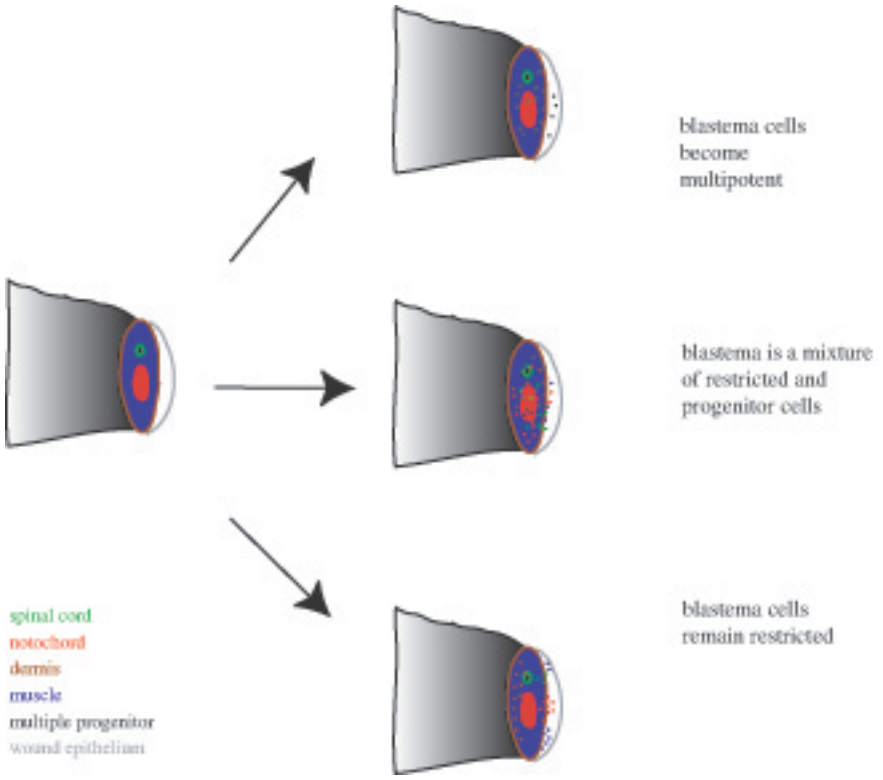


Figure 12. Possible models of blastema cells characteristic. Blastema becomes a pool of multipotent cells with no specific restriction in fate. Second, blastema is a mixture of pluripotent cells and restricted in fate progenitor cells. Third, blastema cells remain restricted to their lineage.

The evidences available so far regarding blastema cells origin allow the prediction, that cells at the amputation plane turn on a cascade of signals driving the mature cells to dedifferentiate (confirmed by the loss of tissue identity markers, e.g. muscle markers) and take up the characteristics of progenitor cells. The pluripotency of these cells is still to be determined. However, the notion directs models towards stem-cell-like dedifferentiation or at least to a high level of pluripotency. Having both in mind, the obvious questions arise: are blastema cells pluripotent? Can the structure of the spinal cord be reconstituted form blastema cells?

In order to address these questions, the experiment was designed where a GFP+, 4 day blastema was transplanted into the place of a removed fragment of the spinal cord (Figure 13A). There was a possibility of the presence of neural progenitors in the blastema. To exclude this, every implanted blastema was divided in half, where one

half of the blastema was implanted into the tail and the second half was sectioned and stained against a glial marker GFAP (Figure 14).

Animals subjected to transplantation (Table 6) were allowed to recover, and after 3, 5 and 8 days the tail was amputated and the part with the transplant was fixed, sectioned and stained against GFAP to examine if GFP+ cells contributed to the structure of the regenerating spinal cord. (Figure 15)

Nr of transplanted animals	*Nr of animals sectioned after 3 days*	*Nr of animals sectioned after 5 days*	*Nr of animals sectioned after 8 days*	*Nr of animals allowed to complete regeneration*
36	9	9	11	7

Table 6. 36 animals were subjected to transplantation of GFP+ blastema into the injured spinal cord. Animals were divided in groups and tails were sectioned at different time points of regeneration. 7 animals were allowed to complete regeneration.

Animals that were allowed to fully regenerate contained GFP+ muscle cells and cartilage (Figure 13B-E). No contribution of blastema to the spinal cord was observed. In most cases, groups of blastema cells were pushed away from the regenerating spinal cord part and located either between the spinal cord and cartilage or on the side of the spinal cord.

To observe the possible contribution of blastema cells to regenerating spinal cord in time, animals were sectioned in three stages of regeneration – 3, 5 and 8 days. Each specimen was sectioned and stained against GFAP. In all cases no single cell positive for both GFP and GFAP was detected. Only several, morphologically definable GFP+ cartilage cells were found (Figure 15 – example at 5 days).

It still remains to be clarified how transplanted cells would populate the regenerating tail if animals were left to grow for a longer period of time (e.g. 2 months after transplantation) or what the fate of those cells would be when they would have been subjected to amputation. The question here is whether the regeneration completes its programme within the first two weeks after injury or if we could call regeneration not only reconstitution of the lost appendage, but also rebuilding of the whole structure including the patterning of muscle segmentation.

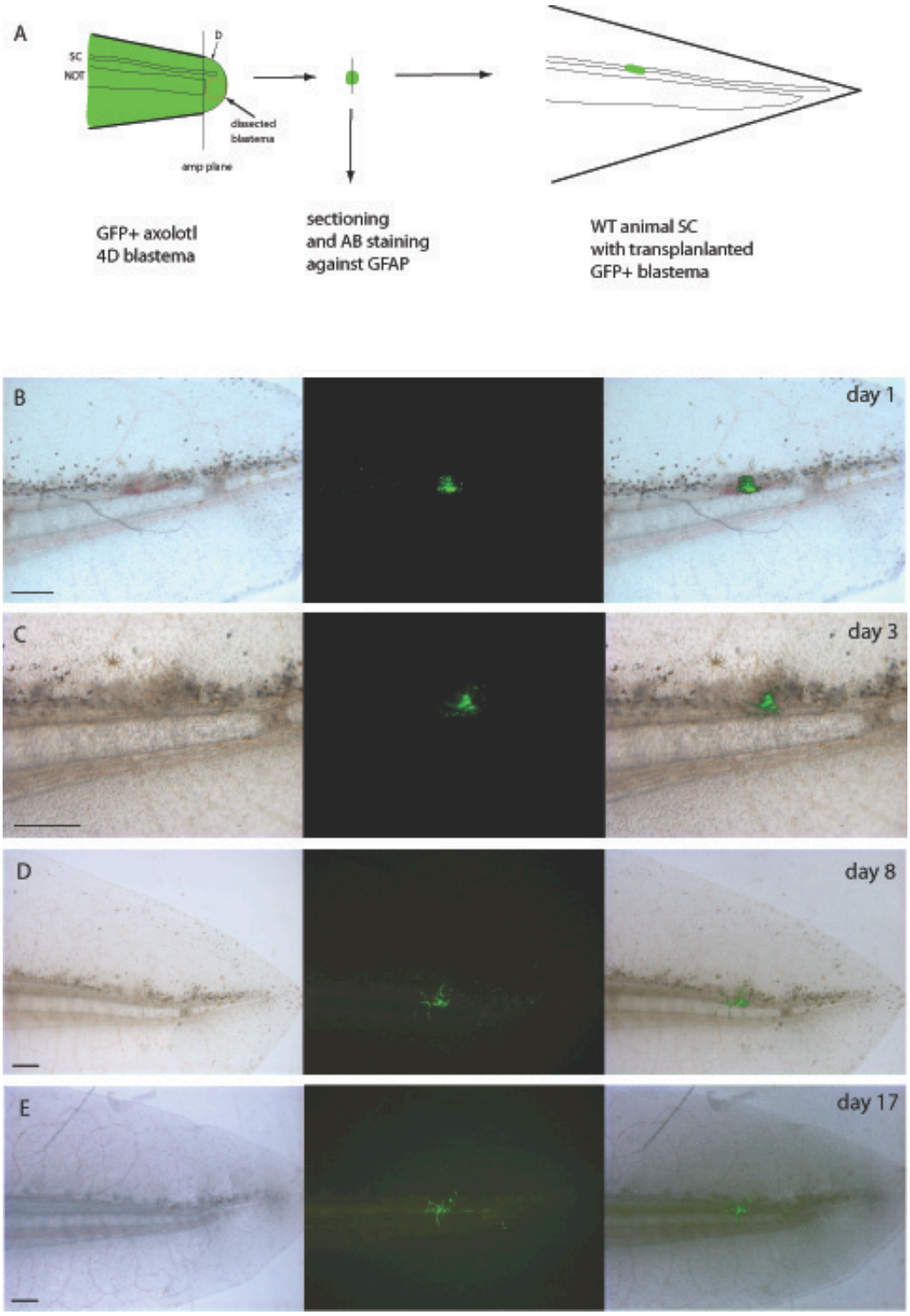


Figure 13. Schematic diagram showing 3 cm long GFP+ axolotl amputated tails, which were allowed to regenerate to the 4-day blastema. (A) Blastema was dissected and divided in two fragments. One fragment was fixed, sectioned and stained with antibody against GFAP (Figure 14). The second fragment was implanted into the injured spinal cord of a *white* mutant 3 cm long axolotl in the middle of the tail. B and E show the regeneration of the representative tail. No contribution of GFP+ cells to the spinal cord was observed. Scale bars: 0.5mm.

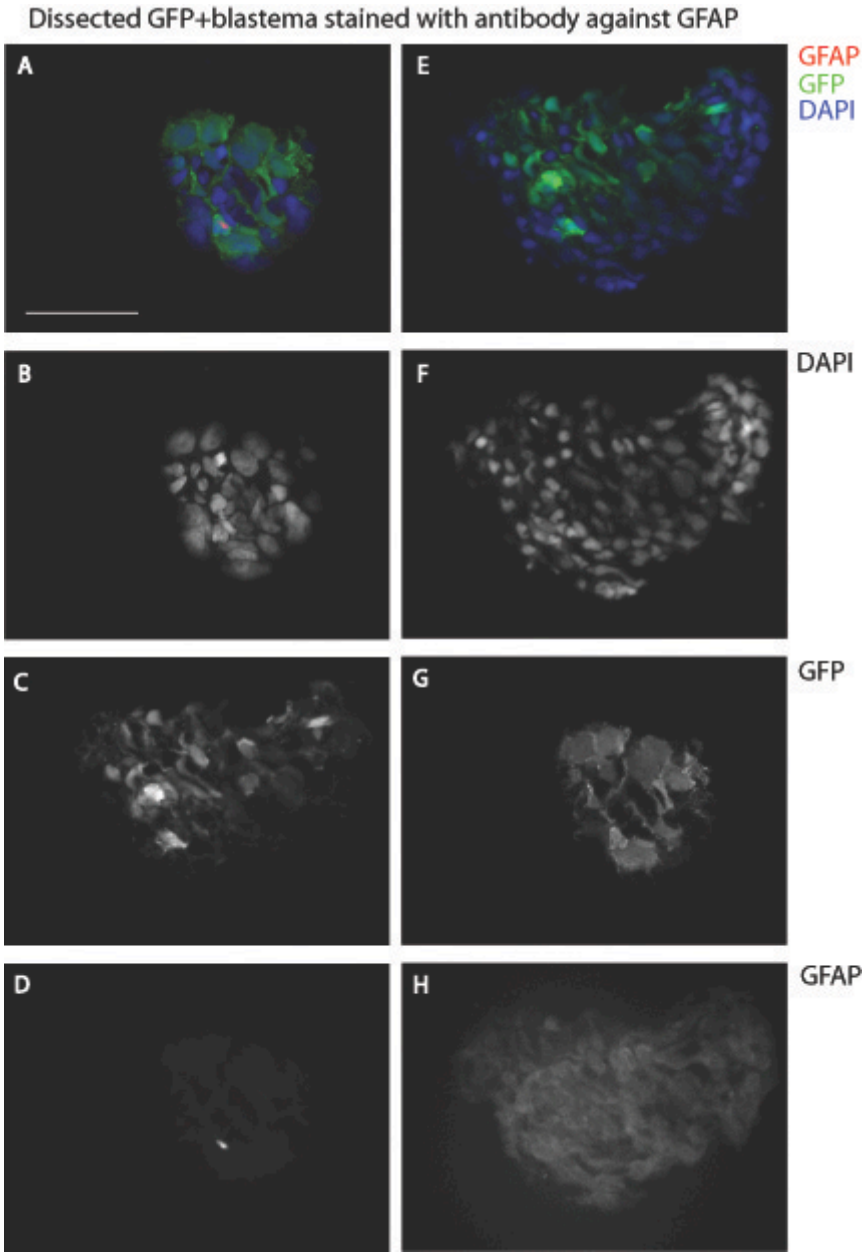


Figure 14. GFP+ blastema was sectioned and stained with antibody against GFAP in order to exclude the possibility of the presence of neural progenitor cells. Panels A-D and E-H show two representative blastemas where no GFAP+ cells were detected. Scale bar: 100 μm.

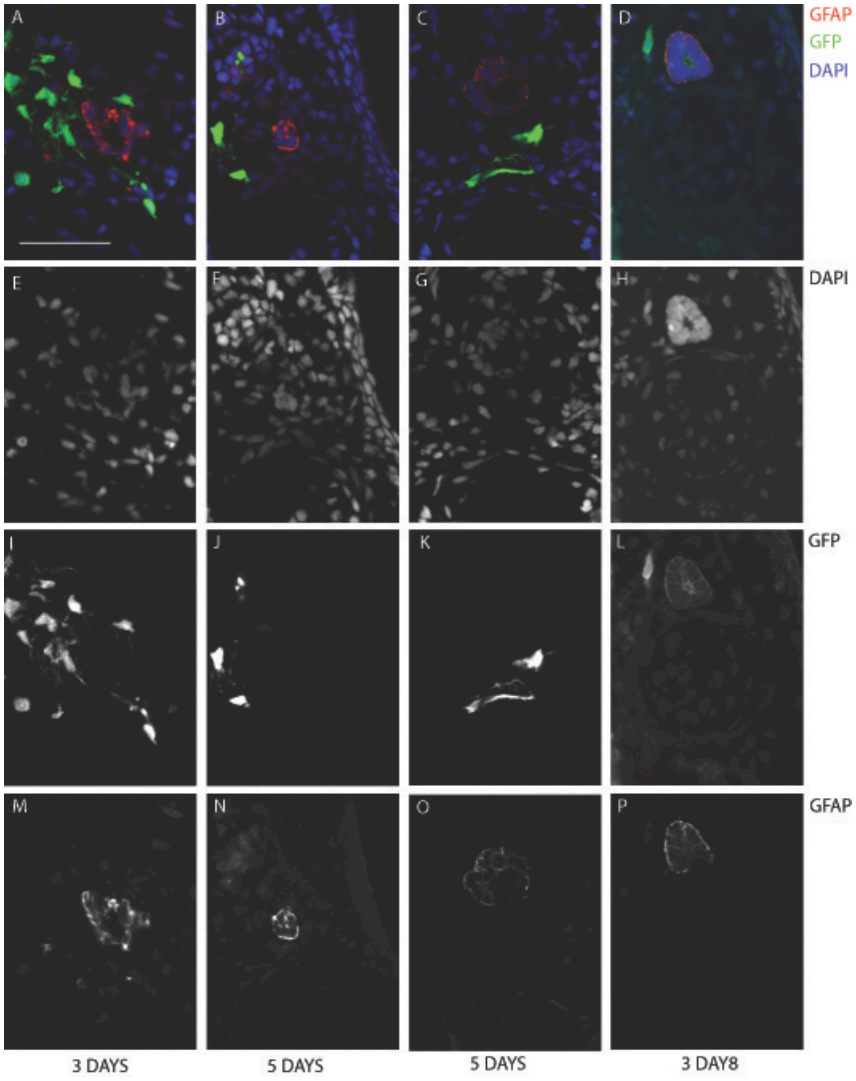


Figure 15. Regions where GFP+ blastema cells were transplanted into the spinal cord were fixed and sectioned at different time points. Sections were stained with antibody against GFAP (M-P). Vertical panels represent four different tails: A, E, I, M – represent the tail sectioned three days after implantation. Spinal cord stained with GFAP (red) is surrounded with GFP+ cells from the transplant. B, F, J, N - represent the tail sectioned five days after implantation. Green cells exited the spinal cord region and are visible in the area of dedifferentiating muscle. C, G, K, O - represent another example of the tail sectioned five days after implantation. Green cells contributed to the regenerating cartilage. D, H, L, P - represent the tail sectioned eight days after implantation. No GFP+/GFAP+ cells were detected. GFP+ cells are visible in the area of muscle. All sections were stained with DAPI to visualize nuclei (E-H).

2.10 Future of transgenesis in the axolotl

We have shown so far that using fairly simple methods it is possible to obtain transgenesis in the axolotl. The great advantage of the GFP+ axolotl over tools available previously, is its stable expression of the GFP protein that can be followed continuously through the whole life of the animal and during regeneration *in vivo*. One advantage of the axolotl system in regeneration research is the possibility to study regeneration on the same animal several times, while collecting the regenerated part for histological analysis and observe the regeneration in the same conditions again.

Experiments described in this dissertation showed that transgenesis in the axolotl is feasible. However, the ultimate transgenesis goal in the axolotl is the implementation of a Cre-loxP system (Nagy, 2000) in order to label tissues and cells specifically and follow the fate of a single cell in the regeneration process. Cre recombinase efficiently catalyzes recombination between two of its recognition sites (loxP sites) flanking the gene in any environment and in any cell. The sophisticated nature of this system is shown on Figure 16.

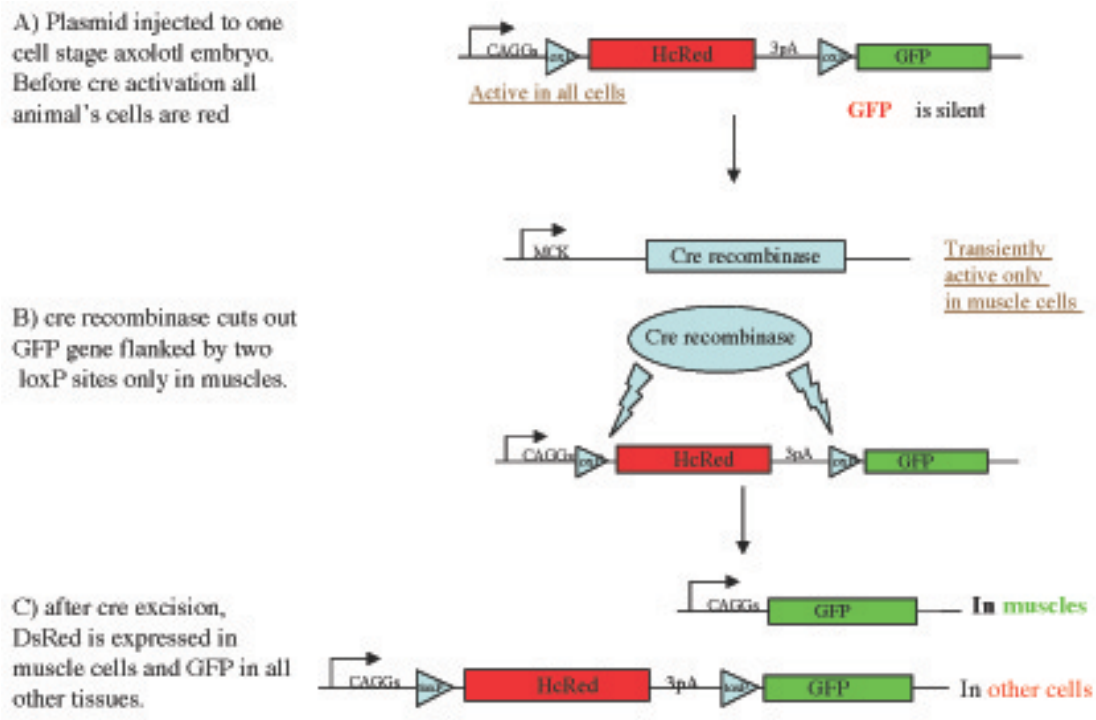


Figure 16. Schematic diagram of Cre-loxP system for implementation in the axolotl system.

Implementation of this system allows conditional manipulation of reporter gene expression and would open numerous possibilities for cell fate tracing and gene expression analysis.

A very interesting problem in regeneration is the origin of blastema cells and their ultimate fate. A great example of the application of the Cre-loxP system in regeneration studies is to reveal the fate of the muscle fiber. Does muscle fiber dies or does it dedifferentiate to populate blastema with progenitor cells? Are those muscle-derived blastema cells restricted in their fate to form muscle again, or upon dedifferentiation do they acquire potency to transdifferentiate? The possible outcomes of muscle cell fate tracing experiment are shown on Figure 17.

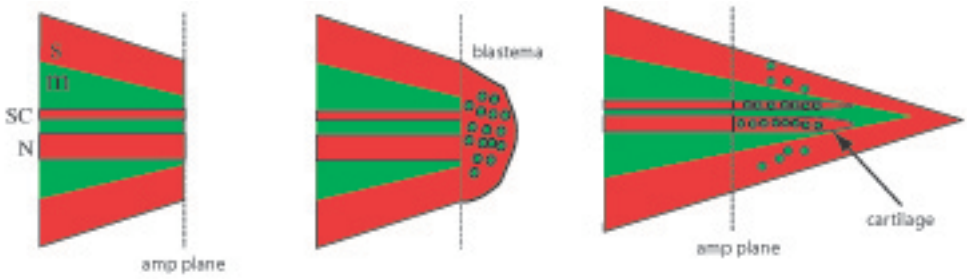


Figure 17. Possible application of Cre-loxP system in transgenesis in the axolotl. Construct pHcRDR is ubiquitously expressed in every tissue of the tail. Upon Cre-mediated recombination in muscle cells, this tissue is clearly distinguishable from the rest of the body. Upon amputation, a blastema is formed, and very likely will contain a mixture of red and green cells. After the complete regeneration we will be able to define what is the fate of muscle fibers in the regenerating tail.

Preliminary experiments to test this system in the axolotl were performed. The construct used for this analysis, pHcRDR, is a double reporter gene, where the CAGGs promoter drives the expression of HcRed (flanked by loxP sites) followed by an inactive GFP sequence. Upon Cre mediated recombination, the HcRed sequence is removed from the transgene, allowing the promoter to drive the expression of GFP

and resulting in the change of colour in the visible marker in the cell(s) where the recombination took place.

In the axolotl, this would allow induction of the expression of GFP only in single cells, whose fate could be further followed during the regeneration of the tail.

To test whether the Cre-loxP system works in the axolotl, we bulk-electroporated a mixture of pHcRDR and MCK-Cre plasmid into the axolotl tail. Animals were allowed to heal and after 3 days expression was analyzed (Figure 18).

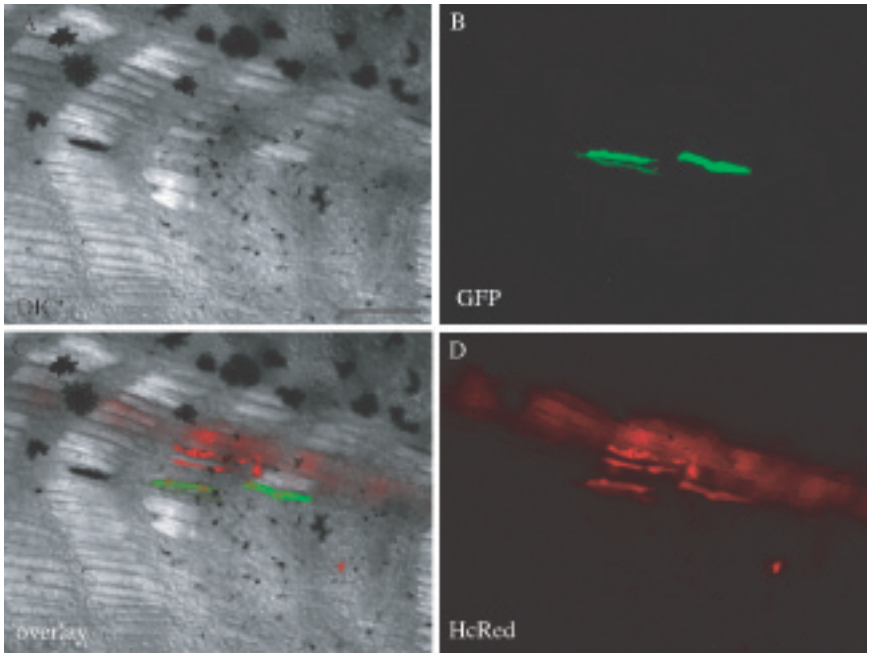


Figure 18. The axolotl tail was electroporated with plasmids pHcRDR and MCK-Cre. After 3 days the transient expression of HcRed was observed in muscle fibers and spinal cord along the tail (D) but GFP expression was only observed in muscles (B) as a result of recombination induced by Cre recombinase. A. DIC image of the electroporation region. C. overlay of DIC, green and red fluorescence. Scale bar - 200μm

We were able to show that Cre recombinase effectively excised the HcRed gene from the plasmid and induced expression of the GFP gene. Although the ability to implement this system in the axolotl is without question, we cannot use electroporation of the pHcRDR plasmid. The expression here was transient and amputation of such tails resulted in the quick loss of the marker and again we were not able to follow the fate of the cells. The only way to answer this question is to produce transgenic a animal ubiquitously expressing a double reporter plasmid, such as pHcRDR, and deliver Cre recombinase to a specific tissue at later stages. In this

way, the expression of the pHcRDR will be stable, will not be lost upon dedifferentiation and will not change or disappear upon the change in a cells identity.

3 DISCUSSION

The ability to produce transgenic axolotls with relative ease opens up numerous opportunities for producing animals harboring other cell markers, and for manipulating gene expression in the axolotl, an important dimension that was previously unavailable for this animal. As shown here, an important aspect is the differing temporal expression profiles of supposedly constitutive promoters, where expression from some promoters was lost over time. Further studies will be needed to determine whether a broad scope of promoters will be functional in these transgenics. In *Xenopus*, faithful tissue specific expression has been observed using promoters from heterologous species (Hartley et al., 2001). We expect that such promoters would display the same tissue specificity using the plasmid injection technique.

While a broader assessment of the transgenic technique regarding different promoters and transgenes is necessary work for the future, an emphasis of the work described here is the use of the GFP germline transgenic animal for cell tracking experiments during development and regeneration. These GFP+ animals are an important tool for embryological and regeneration experiments where specific cell or tissue types need to be followed over long periods of time. Previously, grafting experiments in axolotls were performed using either triploid nucleoli or skin pigment which both have major limitations for analysis (Muneoka et al., 1984; Pescitelli and Stocum, 1980). The pigmentation grafts are only useful in grafts containing skin. In the triploid nucleoli technique, only a proportion of the grafted cells contained three nucleoli and are distinguishable from host cells (Muneoka et al., 1984). Thus, there is not a one-to-one correspondence of grafted cells to the marker. Secondly, the grafts can only be analyzed upon fixation and histological analysis. In contrast, GFP+ tissue can be followed dynamically over time in live animals, and provides an indelible marker with high spatial resolution, as shown here in our studies of the dorsal fin, and of blood during regeneration.

Classical transplantation experiments demonstrated that neural crest cells contribute to the dorsal fin mesenchyme (Raven, 1931; Raven, 1936) and since that time it has been assumed that neural crest is the sole contributor of mesenchymal cells of the dorsal fin (see (Bodenstein, 1952; Tucker and Slack, 2004) Our experiments where we transplant complete GFP+ neural folds indicated that only approximately 50% of the dorsal fin mesenchyme came from neural crest. Transplantation of GFP+ somites at stage 23 resulted in GFP+ dorsal fin mesenchyme, indicating that somites

(presumably dermatome) also contribute cells to the dorsal fin mesenchyme. In light of our new findings, it is possible to re-interpret Bodenstein's (Bodenstein, 1952) transplantation results as being consistent with a dual, neural crest and somite origin of dorsal fin mesenchyme. Bodenstein transplanted prospective dorsal fin epidermis laterally over somites at stage 28 and observed the formation of a proper dorsal fin containing mesenchymal cells. He interpreted this result as indicating that the neural crest cells that had migrated out of the neural tube and populated the somites, had contributed to the ectopic dorsal fin mesenchyme. Our somite transplantations were performed well before neural crest delamination and migration which start in the anterior trunk at stages 27 and 31, respectively, (Epperlein and Lofberg, 1990), eliminating the possibility that the somite-derived fin mesenchyme derives from migrating neural crest. In a second set of experiments, Bodenstein transplanted dorsal tissue that included ectoderm and the dorsal half of the neural tube to the ventral midline in order to observe if these two cell layers were sufficient to form dorsal fin at an ectopic site. The ectopic fins that formed were approximately half the size of the normal fin. When the same dorsal tissue pieces were transplanted over somites, full sized fins formed. In a third set of experiments, when dorsal tissue that included ectoderm, neural tube and somites was transplanted into the ventral midline, full-sized fin structures formed. Thus, there was a strong association of full-sized fin formation with the presence of somites. Our experiments here indicate that this is due to a major contribution of somites to dorsal fin mesenchyme.

An important question for the future is whether the contribution of somites to fin mesenchyme is a feature specific to urodele amphibians or whether it is generally found in all lower vertebrates with fins. Smith and colleagues (Smith, 1994) used DiI labeling to characterize the source of fin mesenchyme in zebrafish. Labeling of neural crest resulted in labeled cells in the dorsal fin mesenchyme, while labeling of somites did not. At this point, it is unclear whether this difference to our results is due to a difference in fin formation between species, or to a technical difference in the experiments.

Here we have also used embryonic transplantation of GFP+ blood anlage to test the frequency and role of hematopoietic cell plasticity during tail regeneration. By transplanting the embryonic blood-forming region, it is possible to label blood cells, including the stem cells that give rise to hematopoietic cells. The axolotl may be an

ideal model system for such experiments, since it is thought that the blood (primitive and definitive) derives solely or largely from the ventral blood islands rather than from additional dispersed sites (Durand et al., 2002). Since the tail is distant from the blood-forming region in the main body, a large graft of the ventral body tissue does not interfere with the specificity of visualizing blood in the tail.

So far we have observed no contribution of labeled blood cells to muscle or central nervous system, as was reported in the mouse (Brazelton et al., 2000; Ferrari G., 1998; Mezey et al., 2000). It is difficult to compare quantitatively the frequency of cell type switching occurring in the mouse with our results. During the course of our experiments we estimate that we have tracked in total approximately 600 GFP+ blood-derived cells (non-circulating), but the total number of stem cells, for example, that we are tracking is unknown. Comparison of GFP+ cells in circulating blood in the germline transgenic animals versus a grafted animal indicated that we had labeled up to 30% of the total blood cell population.

Our results, indicating that blood cells do not contribute significantly to regenerating tissues are consistent with classical irradiation experiments performed to determine whether the regenerating limb blastema arises locally from cells at the amputation plane, or from distant cells (such as circulating blood cells) that would home to the injury site (Butler, 1942). In these experiments, a segment of the left hindlimb was exposed to X-irradiation while the rest of the body was shielded. When the limb was amputated in the exposed region regeneration did not occur, while amputation through the shielded regions did result in regeneration. These experiments indicated that the main proportion of cells involved in limb regeneration arise from the tissue at the amputation plane. Our results coupled with the Butler and O'Brien work indicate that although hematopoietic cells may have the potential to form distant cell types, this plasticity is not utilized or required for regeneration of complex structures such as the limb or tail. Our results would thus imply that a more important issue for understanding regeneration is how tissues at the injury site such as muscle, dermis and nerve are induced to produce the progenitor cells of the blastema.

In the transplantation of blastema cells into the spinal cord we attempted to determine whether blastema cells show the level of plutipotency allowing them to reconstitute every cell type in the tail. So far, it is still not clear whether stem cells residing in the tissue populate blastema upon injury mediated activation and what is the extent of

their contribution to the blastema. We were not able to detect neural progenitor cells in the blastema or blastema cells contribution to the spinal cord, suggesting that neural tissue within regenerating tail mainly reconstitutes itself from the spinal cord structure.

To answer very important for a regeneration research in the axolotl questions of origin of the blastema and fate of mature tissues during regeneration it is of great importance to pursue the development of transgenesis towards implementation of inducible expression systems, such as Cre-loxP system. It would allow manipulations with marker gene expression in desired place in the animal and studying its effect in desired time, e.g. during development or regeneration.

4 MATERIALS AND METHODS

4.1 Animal care, embryo handling and injection protocol

All axolotls were bred and raised in the laboratory. In all experiments, white mutant (d/d) axolotls were used. Axolotls were staged according to the normal tables from (Bordzilovskaya, 1989). In all injection experiments embryos were used at the one cell stage. Embryos were collected in the morning and kept in tap water at 4°C until the time of injection to assure they remained at the one cell stage. All embryos were dejellied manually with fine forceps (Dumont 5, Fine Science Tools, Germany) and kept in 1xMMR with penicillin-streptomycin (Invitrogen GmbH, Germany). Our experience in embryo handling allows us to say that careful manual dejellying does not harm embryos and has very little effect on survival. However, since different spawns of embryos had differing survival rates, one part of each spawn was left as a quality and viability control. Control, non-dejellied, embryos were kept in aerated water.

Prior to injection embryos were transferred to chilled 20% Ficoll/1xMMR/pen-strep. (Ficoll obtained from Sigma, Germany). Injections were performed at the one-cell stage (at 22°C the time from fertilization to the first division is 6 hours; (Bordzilovskaya, 1989). Injections were performed using a pressure injector Picospritzer II (Parker Instrumentation) mounted alongside of an Olympus Stereo SZX12 dissecting microscope. Glass capillary needles were prepared on a Flaming/Brown micropipette puller; model P-97, (Sutter Instruments Co., USA). Needles were back-filled with DNA alone or DNA+ISceI Enzyme (DNA: 0.1µg/µl or 0.01µg/µl; ISceI enzyme: 1U/µl; 1x meganuclease buffer. Enzyme and buffer were obtained from New England Biolabs, USA). 10nl of solution was injected into the one cell stage embryo. The optimal injection volume of 10 nl was established by a series of plasmid injections in the range of volumes from 30nl to 5nl. A volume of 20nl can be used, however it may result in a lower survival rate of embryos depending on their quality. After injection embryos were kept in 20% Ficoll/1xMMR solution for 2-3 hours to prevent yolk leakage and then were transferred to 5% Ficoll/0.1xMMR/pen-strep and left in this solution overnight. Finally, embryos were transferred to 0.1xMMR/pen-strep, until the hatching stage, when animals were kept in aerated tap water at room temperature. All solutions used for injections and embryo rearing were 0.22μm filtered.

4.2 Grafting GFP+ neural folds and somites

Before being used for grafting white mutant (d/d) GFP+ donor embryos and white hosts were washed thoroughly with tap water and sterile Steinberg solution (Steinberg, 1975) containing antibiotics. They were then decapsulated manually. For grafting GFP+ trunk neural fold material or somites, GFP+ donors and hosts were placed into the depression of an agar dish filled with cold Steinberg saline. Grafting was performed under sterile conditions using tungsten needles.

4.2.1 A. Neural folds

Neural folds containing cranial and trunk regions (n=2), or one complete left trunk neural fold (n=8) were grafted orthotopically from white GFP+ transgenic neurulae (stages 15-17) into white mutant hosts from which neural fold material of equivalent size had been extirpated. Two to four days after the operation the distribution of GFP+ cells in the hosts was followed under fluorescence optics.

4.2.2 B. Somites

For grafting single somites, the epidermis was lifted from the left dorsolateral midtrunk of white GFP+ transgenic embryos (stage 23/24). One somite was extirpated and implanted into a similar site of a white host where a somite had been removed. The epidermis was then folded back over the implanted somite and pressed against it with a small glass disc for a few minutes. Migration of GFP+ cells into the dorsal fin was followed under fluorescence optics.

4.3 Grafting GFP+blastema into spinal cord.

Tails of 3cm, GFP+ axolotls (n=37) were amputated and allowed to regenerate for 4 days. Blastema was dissected and divided in two parts. One part was fixed, sectioned and stained with GFAP (company) antibody. The skin of white mutant animals (n=37) was peeled off and spinal cord was injured by removing a small fragment of spinal cord from the middle of the tail (Fig.13). Second part of the dissected GFP+ blastema was implanted in a place of injury and the wound was covered with the skin.

Animals were allowed to recover in aerated water with antibiotics (Pen-strep , Sigma, Germany) at room temperature. During surgerical procedures animals were anesthetized with 0.001% ethyl-*p*-aminobenzoate (Sigma, Germany). Tails of transplanted animals were collected after 3, 5, 8, 17 days and fixed, sectioned and stained against GFAP.

4.4 In Vivo Electroporation of axolotl tails.

The electroporation procedure was performed according to Echeverri and Tanaka (Echeverri and Tanaka, 2003). Animals were anesthetized with 0.001% ethyl-*p*-aminobenzoate (Sigma, Germany) and placed on a platform. The skin was removed, to allow the microinjection of the plasmid mix. Plasmids were use in the concentration 0.1ug/ul and several nanoliters were injected (using World Precision Instrument pressure injector) into the tissue. In the case of this experiment it was desired to express the pHcRDR construct in as different population of cells as possible, therefore plasmid mix was injected into muscles, cartilage and spinal cord. Using SD9 Stimulator (Grass Telefactor) the tail was electroporated with 5 pulses, 200Hz with the 20msec pulse length. The electroporated tail was covered back with the skin, and animals were allowed to recover in 0.1xMMR with antibiotics.

4.5 Histology and immunofluorescence.

Several days after grafting GFP+ tissues larvae were anaesthetized with MS 222 (Serva) and fixed with 4% paraformaldehyde (PFA) in 0.1 M PBS overnight. Fixed specimens were washed and transverse vibratome sections (100 µm) were cut on a vibratome series 1000 sectioning system (Ted Pella, Inc.) in order to investigate the internal distribution of GFP+ cells. Some PFA-fixed larvae of similar age as larvae with GFP+ somites (stage 38) were embedded in paraffin (Leica ASP 300) for investigating the distribution of PAX7 positive dermatome cells on transverse sections (5 µm). Microwaved transverse sections (5 µm) were stained with a primary PAX7 antibody (DSHB) and with biotinylated secondary antibodies and tertiary streptavidin-peroxidase complexes (Vectastain ABC-Elite-Kit).

In the samples for characterizing the fully GFP transgenic animal, 5 day blastemas of GFP+ juveniles, were fixed in 4% PFA overnight at 4°C, washed 3x15min with PBS at room temperature and placed in 30% sucrose in PBS overnight at 4°C. Samples were mounted in Tissue Tek (Sakura) and cryosections (10 μm) were collected on Histobond Adhesion microslides (Marienfeld, Germany). To visualize the structure of the tissues, sections were stained with a hematoxylin solution (Sigma, Germany) for 20 seconds and washed extensively with tap water, followed by a 3-minute wash in PBS. Nuclei were stained for 8 minutes with Hoechst followed by 3x5min washes in PBS. Slides were mounted using Mowiol and allowed to dry over night.
In the experiment of transplantation of 4 day GFP+ blastema into the spinal cord, both dissected blastemas and tails with implanted blastemas were fixed and sectioned as described above. To visualize GFAP+ cells samples were washed several times with 1xPBS/0.3%Tween, blocked with 10%FBS/1xPBS form 10 min and 10%FBS/1xPBS/0.3%Tween for 20 minutes, washed once with PBS, placed in GFAP antibody solution for 1 hour in 37°C, washed 3x5 minutes in 1xPBS/0.3%Tween, covered with secondary antibody TRITC for 1 hour at 37°C, followed by washing steps – 3x10minutes in 1xPBS/0.3%Tween. Nuclei were visualized 1ug/ml Hoechst solution for 10 min, followed by additional washing 2x5min in PBS, and 1x5min in water. Slides were mounted in Mowiol.

4.6 Microscopy

For transgene expression analysis animals were anesthetized with 0.001% ethyl-*p*-aminobenzoate (Sigma, Germany), placed on a coverslip and imaged using a 10x Plan-Neofluor objective on a Zeiss Axiovert 2 microscope with a CCD camera (Diagnostic Instruments, USA) controlled by a Metamorph image acquisition system (Visitron). For imaging of dissected GFP+ organs and tissues we used an Olympus Stereo SZX12 microscope with fluorescence attachment controlled by Diagnostic Instruments software (USA). Sections of GFP+ tissues from transgenic GFP animals were imaged on an Olympus BX61 microscope using brightfield and fluorescence optics.

4.7 Plasmid constructs

Plasmid constructs used in experiments were: pCSKA-hGFP-SceI (5.0kb) (kindly provided by C. Grabher) that contains the *Xenopus borealis* cytoskeleton actin promoter driving hGFP flanked by SceI sites. CMVGFP3-ITR (5.3kb). pCS2nβgalITR was digested with HindIII and Xho1 to remove the lacZ sequence, which was replaced by the GFP3 sequence obtained after HindIII/XhoI digestion of XCarGFP3ITR. GFP3 is a version of GFP that was mutated for better expression in *Xenopus* (both plasmids used for this cloning - kind gift of Sylvia Evans).

pCAGGsEGFP(Sce) (5.9kb) was constructed by insertion of two oligonucleotides containing ISceI recognition sequences in opposite orientations flanking the pCAGGsEGFP cassette that contains EGFP driven by chicken β-actin promoter with CMV-IE (CAGGsEGFP plasmid kindly provided by F. Stewart).

Plasmid pHcRDR was a kind gift of Dr. Frank Eddenhoffer, MCK3.3-Cre plasmid was kindly provided by Rico Barsacchi.

Plasmids were purified using MaxiPrep Kits (Quiagen, Germany) (pCSKA-hGFP-SceI, pCAGGsEGFP(SceF), MCK3.3-Cre and CMVGFP3-ITR), EndoFree Quiagen Kit (pCAGGsEGFP and pCAGGsEGFP(Sce)) and cesium chloride preparation (pCAGGsEGFP(Sce), pHcRDR). The type of plasmid purification in meganuclease injections did not seem to have a significant influence on expression or survival.

The *ISceI* meganuclease enzyme (NEB, Germany) was aliquoted and stored at –80°C and used according to the teleost protocol available by personal communication with Jochen Wittbrodt and Clemens Grabher (Jochen.Wittbrodt@EMBL.de or Clemens.Grabher@EMBL.de).

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7 ABBREVIATIONS

AAV ITR	Adeno-associated Viral Inverted Terminal Repeats
CAGGs	promoter: chicken beta actin + CMV IE enhancer
CMV	Cytomegalovirus
CSKA	cytoskeletal actin
F0,F1	generation of transgenic animals – F0 being the founder, F1 – first generation of progeny
GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
MCK	Muscle Creatine Kinase
MMR	Mark's Modified Ringers
PFA	paraformaldehyd
PBS	Phosphate Buffered Saline
REMI	Restriction Enzyme – Mediated Integration

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9 PUBLICATIONS

Sobkow, L., Epperlein, HH., Herklotz, S., Straube, W., Tanaka, E.M., "A germline GFP transgenic axolotl and its use to track cell fate: dual origin of the fin mesenchyme during development and the fate of blood cells during regeneration" **–** Developmental Biology, 2006 Feb 15;290(2):386-97

I herewith declare that I have produced this paper without the prohibited assistance of third parties and without making use of aids other than specified; notions taken over directly or indirectly from other sources have been identified as such. This paper has not previously been presented in identical or similar form to any other German or foreign examination board.

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